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Transitions between phases of genomic differentiation during stick-insect speciation

Rüdiger Riesch[1*], Moritz Muschick[2*], Dorothea Lindtke[3*], Romain Villoutreix[3*], Aaron A. Comeault[4], Timothy E. Farkas[5], Kay Lucek[3], Elizabeth Hellen[3], Víctor Soria-Carrasco[3], Stuart R. Dennis[6], Clarissa F. de Carvalho[3], Rebecca J. Safran[7], Cristina P. Sandoval[8], Jeff Feder[9], Regine Gries[10], Bernard J. Crespi[10], Gerhard Gries[10], Zach Gompert[11*], and Patrik Nosil[3]

[1]School of Biological Sciences, Royal Holloway, University of London, Egham, TW20 0EX, UK
[2]Department of Fish Ecology & Evolution, EAWAG Centre for Ecology, Evolution and Biogeochemistry, 6047 Kastanienbaum, Switzerland
[3]Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN, UK
[4]Department of Biology, University of North Carolina, Chapel Hill, North Carolina, 27599, USA
[5]Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut 06369, USA
[6]Department of Aquatic Ecology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600 Dübendorf, Switzerland
[7]Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, Colorado, 80309, USA
[8]Department of Ecology, Evolution, and Marine Biology, University of California, Santa Barbara, California, 93106, USA
[9]Department of Biology, Notre Dame University, South Bend, Indiana 11111, USA
[10]Department of Biological Sciences, Simon Fraser University, Burnaby, BC V5A 1S6, Canada
[11]Department of Biology, Utah State University, Logan, Utah 84322, USA

*These authors contributed equally to this work.

Short title: **Genomics of speciation**

Speciation can involve a transition from a few genetic loci that are resistant to gene flow to genome-wide differentiation. However, only limited data exist concerning this transition and the factors promoting it. We study phases of speciation using data from >100 populations of 11 species of Timema stick insects. Consistent with early phases of genic speciation, adaptive colour-pattern loci reside in localised genetic regions of accentuated differentiation between populations experiencing gene flow. Transitions to genome-wide differentiation are also observed with gene flow, in association with differentiation in polygenic chemical traits affecting mate choice. Thus, intermediate phases of speciation are associated with genome-wide differentiation and mate choice, but not growth of a few genomic islands. We also find a gap in genomic differentiation between sympatric taxa that still exchange genes and those that do not, highlighting the association between differentiation and complete reproductive isolation. Overall, our results suggest that substantial progress towards speciation may involve the alignment of multi-faceted aspects of differentiation.

Speciation involves genetic differentiation[1-3]. In the absence of gene flow, genome-wide differentiation can readily build by selection and drift. Differentiation with gene flow is potentially more complex, as the homogenising effects of gene flow must be countered[1-3]. The genic model of speciation proposes that specific genetic regions subject to strong divergent

natural or sexual selection become resistant to gene flow (i.e., exhibit 'reproductive isolation', RI) before others[4,5]. This model thus predicts localised, and potentially few, regions of accentuated differentiation or 'genomic islands' at the initiation of speciation[1,6]. It also predicts that genes subject to divergent selection reside in regions of accentuated differentiation. Consistent with such patterns, colour-pattern differences between sub-species of crows and races of butterflies map to a few localised peaks of genetic differentiation[7-9].

As speciation progresses, additional genetic regions differentiate and the effects of RI become more genome-wide[1,3-5], either because genomic islands grow, background differentiation lifts, or a combination of these processes. Differentiation need not be uniform as, for example, regions experiencing particularly strong selection or reduced recombination still exhibit the greatest differentiation[1,3,10]. Nonetheless, widespread differentiation is predicted in this 'genomic' phase of speciation. Evidence for divergent selection promoting this process (rather than genome-wide drift) is bolstered if: (1) gene flow is still appreciable, (2) genome-wide differentiation is correlated with environmental differences or traits under divergent selection (i.e., genome-wide 'isolation-by-adaptation', IBA)[11,12], and (3) genome-wide responses to selection are confirmed with experiments[13-15]. Genome-wide differences have been documented in herring[16], mosquitoes[17], and apple-maggot flies[10,14], and genome-wide IBA has been reported in many organisms[11,12]. Notably, theory predicts genomic differentiation can be promoted by polygenic adaptation[3], epistasis[18], the coupling of differentiation across loci (as in hybrid zone theory)[19], and mate choice[20,21].

Genic and genomic phases of speciation represent extremes on a quantitative spectrum where differentiation transitions from localised to genome-wide (Fig. 1). This view is consistent with many models of speciation, and with the biological species concept[2,3,22-24]. Indeed, RI eventually becomes a property of the entire genome[25]. Although this spectrum provides a conceptual and theoretical framework for analysing speciation[1,3-5,19,26], empirical understanding of it is limited. This is because replicated genomic studies across the spectrum are still restricted to a few systems such as cichlid fish[27], stickleback[28], flycatchers[29], and Heliconius butterflies[30] (reviewed by[1]). Work on these systems suggests that localised differentiation is promoted by divergent selection and reduced recombination, but that genome-wide differentiation can evolve early in speciation[1,27-30]. However, uncertainties remain about underlying speciation processes and the role of genomic islands[23,26,31]. Additional studies of phases of genomic differentiation are required, especially if generalities are to be established.

Here we study genomic differentiation in Timema stick insects, testing the predictions described above (Fig. 1). We report localised differentiation associated with colour-pattern loci. We find a transition to genome-wide differentiation despite gene flow, associated with mate choice. Indeed, we observe appreciable genome-wide differentiation in sympatry (e.g., mean F_{ST} ~ 0.10, ranging up to 0.27). However, we find little evidence for the growth of genomic islands and report that maximal differentiation is associated with a lack of measurable gene flow. The context-dependent nature of the results renders arguments about the 'importance' of the above factors somewhat subjective; different factors affect different aspects of differentiation (Fig. 1).

Our data also quantify the 'speciation continuum'. A fairly uniform speciation process should leave an observable and inter-connected continuum of populations varying in differentiation[32], a pattern now reported in plant and animal taxa[1]. For example, pea aphid host races vary quantitatively in levels of genetic differentiation[33], and natural hybridisation between butterflies declines gradually with genetic distance[34]. However, theory predicts that speciation

can also be a less uniform process with variable dynamics across time or space, due to changes in gene flow, sudden coupling of differentiation across loci[3], waiting time for mutations[18], non-linear accumulation of genetic incompatibilities[18,35], and rare founder events[25]. If such dynamics cause sudden increases, decreases, or halts in the accumulation of differentiation, then 'gaps' in the speciation continuum may be observed. With sufficient sampling, such gaps can be recognised by a paucity of intermediate forms (i.e., bimodal distributions). The frequency and causes of gaps remain open questions, which we help address here.

Study system, background, and approach

Timema are wingless, plant-feeding insects found in South-western North America[36]. Previous work in T. cristinae has shown that divergent selection between conspecific populations on different host plants (ecotypes hereafter) promotes adaptive differentiation, most markedly in colour-pattern traits conferring crypsis against visual predators[37,38]. Ecotypes also exhibit mate choice and partial sexual isolation, but this is not based on colour-pattern[37,38] (Figs. 1, 2). Several studies have shown substantial gene flow between T. cristinae ecotypes[13,39]. Specifically, there are some 50 migrants per generation ($N_e m$) in populations found in the same locality and ~5-10 $N_e m$ in populations separated by 1-10 kilometres (km)[13,39]. As in most other systems[1,2], the dynamics of speciation from its onset to end are unresolved.

We use data from thousands of individuals from >100 host-plant-associated populations of 11 sexual Timema species to tackle this issue. Our study includes genomic data suitable for population level analyses and genome-wide association (GWA) mapping, such as genotyping-by-sequencing (GBS) data, and low-coverage whole-genome re-sequencing data from >1000 individuals (see Methods, Fig. S4). There are four aims: (1) testing if genetic regions harbouring colour-pattern loci exhibit accentuated genetic differentiation between T. cristinae ecotypes, (2) testing if differentiation in traits affecting mate choice associates with sexual isolation and genome-wide differentiation in T. cristinae, (3) quantifying genomic patterns of differentiation in multiple Timema ecotypes and species, and (4) examining the time course to complete RI.

In past work, genetic differentiation between T. cristinae ecotypes was quantified at the fine scale of single nucleotide polymorphisms (SNPs)[13]. This approach revealed numerous modest-size regions (i.e., thousands of base pairs) of accentuated and parallel differentiation that were spread across linkage groups (LG). A between-generation transplant-and-sequence experiment showed that these regions were statistically enriched for regions likely affected by divergent selection between hosts. Thus, previous work already suggests that divergent selection promotes fine-scale differentiation across many genetic regions during the early phases of speciation. Here our interest is in the transition to larger-scale differentiation. Thus, rather than analysing SNPs we estimated differentiation metrics (e.g., F_{ST}) in 20-kilobase (kb) windows and used a Hidden Markov Model (HMM)[40] approach to assign windows to larger, contiguous regions of accentuated or background differentiation. This means that our results concern large genomic blocks (or in other cases, mean genome-wide differentiation). Fine-scale differentiation exists for individual SNPs, or clusters of them, even in cases where blocks of accentuated differentiation are not detected.

Given subtle allele frequency differences and high gene flow between conspecific ecotypes our whole genome analyses of within-species variation focus on F_{ST}. Indeed, genome-wide differentiation between ecotypes studied here is sufficiently weak that D_{XY} is near perfectly correlated to nucleotide diversity, i.e., π (for all conspecific ecotype pairs the correlation

between D_{XY} and π is >0.99, Pearson correlation). Thus, D_{XY} within species effectively measures diversity, not differentiation. We do report patterns of D_{XY} when considering whole genomes of species pairs, because of their strong differentiation. We note that our conclusions side against speciation being associated with one or a few islands of differentiation. Thus, most criticisms of the use of F_{ST} to study speciation do not apply, because these criticisms are based on the argument that F_{ST} over-estimates the importance of genomic islands for reduced gene flow[31]. Also, F_{ST} as an estimate of genome-wide, rather than localised, differentiation is not subject to these criticisms. As described below, we use analytical tools in addition to F_{ST} to bolster inferences (e.g., Approximate Bayesian Computation, GWA mapping, model-based analyses of genetic structure, phylogenetic inference).

RESULTS AND DISCUSSION

Colour-pattern loci are associated with localised genetic differentiation

We tested if loci affected by divergent selection exhibit accentuated differentiation between Ceanothus and Adenostoma host-plant ecotypes of T. cristinae. We consider a colour-pattern trait (a white dorsal stripe) that is subject to divergent natural selection between these hosts due to visual predation[41] (Fig. 2). GWA studies within a polymorphic population and genetic crosses have shown that this trait is largely controlled by one or few regions on LG8[42]. However, differentiation of this region between ecotypes in nature is untested.

We found three lines of evidence that divergent selection on colour-pattern promotes localised differentiation (Figs. 2, S2). First, we sampled a geographic cline that transitions from an area dominated by Ceanothus to one dominated by Adenostoma. Based on 1598 individuals collected across 33 sites we inferred allele frequencies from phenotype frequencies using knowledge of the genetic basis of colour-pattern[42] (Fig. S3, Table S10). We found a steep cline in colour-pattern allele frequencies, with some analyses showing near fixed differences at a distance of ~5 km. Genome-wide differentiation between ecotypes is weak at this distance (F_{ST} ~ 0.03)[13]. Although this evidence is indirect, it suggests colour-pattern loci overcome gene flow more strongly than the remainder of the genome.

Second and more directly, we found that SNPs associated with colour-pattern reside in regions of accentuated differentiation between ecotypes. Using published data[42] and GWA analyses, we mapped colour-pattern (% dorsal body area striped) and confirmed that SNPs strongly associated with this trait were restricted to LG8. Using 160 previously published genomes[13] we estimated regions of accentuated F_{ST} between four ecotype pairs with the HMM[40] approach. We detected such regions for only two of the four pairs, and they were only modestly elevated over background levels. This finding suggests that gene flow has strong homogenising effects at the scale of the large genomic blocks analysed here. Nonetheless, SNPs associated with colour-pattern coincide with HMM regions of accentuated differentiation between ecotypes ~12× more often than expected by chance ($P = 0.0033$, randomisation test).

Third, a within-generation transplant-and-sequence experiment using 473 new whole genomes from T. cristinae revealed that the highest concentration of genetic differentiation between populations transplanted to different hosts occurred on LG8 (Fig. 2). Thus, the observed number of windows assigned to the high differentiation state on LG8 was ~2-3x greater than expected by chance (observed = 164, null = 63, $P < 0.001$, randomisation test). Nonetheless, we did observe differentiation on other LGs. Coupled with past SNP-based analyses[13], the

1 results suggest that divergent selection promotes differentiation of modest-sized regions on
2 multiple LGs[13] and larger-scale differentiation on the LG containing colour-pattern loci.
3
4 Colour-pattern loci are not associated with genome-wide differentiation
5
6 We next tested for associations between trait differentiation and mean genome-wide F_{ST} (i.e.,
7 genome-wide IBA). We did so using GBS data for 21 pairwise comparisons for which data
8 exist also on sexual isolation[43]. These populations occur at the 1- to 10-km scale of restricted
9 but non-zero gene flow. After controlling for geographic distance, we found no evidence that
10 population differentiation in colour-pattern has an effect on mean genome-wide F_{ST} (posterior
11 probability that the effect was > 0, pp hereafter, was < 0.60, n = 21, Bayesian linear mixed
12 model, BLMM). Thus, effects of colour-pattern on genetic differentiation are localised in the
13 genome, consistent with this trait being largely controlled by a single LG and that it does not
14 affect mate choice[44].
15
16 CHC variation and its genetic basis
17
18 We next studied cuticular hydrocarbons (CHCs). We did so because CHC differentiation is
19 inversely correlated with mating probability between Timema species[45], and CHCs affect mate
20 choice in other insects[46]. Thus, CHCs could affect genomic differentiation. We quantified the
21 genetic basis of CHCs, and tested their association with mate choice and genomic
22 differentiation.
23
24 We quantified three classes of CHCs and found strong sexual dimorphism (sex effect, $F_{6,334}$ =
25 56.86, P < 0.001, Wilks' partial η^2 effect size = 50.5; host-plant effect, $F_{6,334}$ = 13.90, P <
26 0.001, partial η^2 = 20.0; MANOVA, Fig. 3). We thus quantified the genetic architecture of
27 CHCs in males and females separately. GWA mapping supports a polygenic basis to CHCs
28 with a modest but non-zero heritability. We observed a correlation between the number of
29 CHC-associated SNPs per LG and LG size (r > 0.99, P < 0.01, for all six combinations of two
30 sexes and three CHC classes, i.e., 'traits', Fig. 3). This pattern argues against major locus
31 control, but could arise if CHCs were completely non-heritable or via heritable variation with
32 polygenic control[47]. We distinguished these alternatives by testing if CHC variation was
33 partially explained by genotype, which would support non-zero heritability. Consistent with
34 this hypothesis, we found that estimates of the median percent variance explained (PVE) by
35 genotype were ~30% in females and ~60% in males, albeit with wide credible intervals around
36 these point estimates (Figs. 3, S2, Table S6-8 for details). Moreover, we detected low but
37 significant predictive power in cross-validation (i.e., genomic prediction) analyses for five of
38 six CHC traits (Table S8 for details). Low predictive power is expected for polygenic traits[48],
39 but even limited predictive power strongly suggests non-zero heritability.
40
41 CHCs and mate choice
42
43 We conducted perfuming experiments and found that female CHCs causally affect mate choice
44 within a population of T. cristinae and sexual isolation between a species pair (treatment
45 effects, Log Rank: X^2= 28.211, P < 0.001; all post-hoc pairwise comparisons, P < 0.01, Fig. 3,
46 Table S9). As recently reported for Drosophila CHCs[46], the relation between mate choice
47 within species and sexual isolation is not necessarily straightforward. Although we do not
48 know for certain the extent to which female CHCs cause sexual isolation between conspecific
49 populations (this was not tested experimentally), some effect seems likely given that the
50 perfuming experiments show causal effects on mate choice within species and sexual isolation

between species, and given that population differentiation in female CHCs in T. cristinae is positively correlated with degree of sexual isolation (partial coefficient controlling for geographic distance = 0.08, pp = 0.97; partial coefficient controlling for genome-wide F_{ST} = 0.08, pp = 0.96, n = 21, BLMM, Fig. 3).

In contrast, male CHCs seem not likely to affect mate choice. This is because males choose females as mates in Timema[49], and population differentiation in male CHCs is not correlated with sexual isolation (partial coefficient controlling for geographic distance = -0.02, pp = 0.38; partial coefficient controlling for genome-wide F_{ST} = -0.02, pp = 0.38, n = 21, BLMM).

CHCs are associated with genome-wide differentiation

CHCs in T. cristinae appear polygenic. The effects of polygenic traits on genomic differentiation are difficult to predict. On the one hand, their differentiation affects many genetic regions. On the other, their differentiation may be difficult to achieve with gene flow, due to weak per locus selection coefficients[6]. We found that population differentiation in female CHCs was positively correlated with mean genome-wide F_{ST} after controlling for geographic distance (partial coefficient = 0.13, pp = 0.99, BLMM; Fig. 3). In contrast, differentiation in male CHCs was not (pp < 0.60). As for the analyses with colour-pattern, the populations examined occur at the 1- to 10-km scale of restricted but non-zero gene flow. Thus, an association of polygenic traits with mate choice might be important for genome-wide differentiation with gene flow. However, the correlational nature of this analysis urges future work on causal associations between trait divergence, gene flow, and genetic differentiation.

Estimates of heritability (i.e., PVE) of female CHCs were modest but non-zero. Thus, their association with RI and with genomic differentiation likely involves genetic factors. Nonetheless, environmentally induced effects almost certainly contribute, as for most quantitative traits[48]. Induced effects on RI have been reported for imprinting of song in birds[50], cultural differences among killer whale ecotypes[51], and host or mate preference in insects[52]. On the other hand, if environmental effects can be reversed, this could decrease RI. Further work on the role of genes versus induced effects in speciation is warranted. We next tested if the localised and genome-wide differentiation observed in T. cristinae was representative of that in the genus broadly, and of potentially more advanced phases of differentiation.

Genomics of the speciation continuum in Timema

We collected whole-genome re-sequence data from 379 Timema across 10 taxon pairs. Eight pairs were conspecific ecotypes (within six species) and the other two a species pair within two localities. Half of the conspecific ecotype pairs examined exhibit a few HMM regions of accentuated differentiation, which were usually only modestly elevated above background levels (Tables S4, S5). The other half lacks such regions. We found some variation in genome-wide F_{ST} among comparisons, but this appeared unrelated to the presence or number of regions of accentuated differentiation (Fig. 4). Approximate Bayesian Computation (ABC) and island-equilibrium analyses support gene flow between all conspecific ecotype pairs (Fig. S1).

These results imply that the early to moderate phases of Timema speciation involve more than just growth of a few islands of differentiation. Rather, localised genetic changes may be associated with only restricted progress towards speciation unless they align with mate choice, other forms of RI, or factors promoting genome-wide differentiation (e.g., geographic separation). Indeed, the species pair (T. poppensis and T. californicum) showed both strong

genome-wide differentiation and multiple regions of accentuated differentiation (Fig. 4, Tables S4, S5; D_{XY}, locality SM: background = 0.00116, accentuated = 0.00203, 23 accentuated regions with a mean size of 374.8 20-kb windows, equalling 33.8% of the 20-kb windows; locality LP: background = 0.00115, accentuated = 0.00199, 20 accentuated regions with a mean size of 445.9 20-kb windows, equalling 35.0% of the 20 kb windows). We suspect the alignment of multi-faceted aspects of differentiation could be important for speciation in many systems where RI evolves in a polygenic fashion.

Mean genome-wide differentiation between sympatric ecotypes

Because genome-wide differentiation appears common in Timema, we quantified the extent of it when the potential for gene flow is high (i.e., sympatry). We estimated genome-wide F_{ST} based on GBS data obtained from sampling across the geographic and host range of 11 Timema species at 47 localities (n = 1505 specimens)(Fig. 5, Table S1). This yielded 89 within-locality comparisons ('sympatry'). Sixty of these were between conspecific host ecotypes, and 29 between three different pairs of species. This sampling covers most variation in geographic range and host use in these species, and includes all of the known sympatric sexual species pairs in the genus[36].

We observed a continuum of differentiation among sympatric ecotypes, with genome-wide F_{ST} ranging from 0.03 to 0.27 (mean = 0.09)(Fig. 5). The upper end of differentiation is thus appreciable, but never exceeded 0.30. Model-based analyses in ENTROPY[53] support gene flow and admixture between sympatric ecotypes (Fig. S1; Tables S2, S3). The geographic potential for gene flow was similar among ecotypes (i.e., all comparisons are sympatric). Thus, variation in genome-wide F_{ST} likely reflects, in part, the strength of RI. However, other factors such as demographic variability, time in geographic contact, and subtle variation in arrangement of host-plants most likely contribute.

A gap in genomic differentiation restricted to sympatry

In contrast to ecotypes, mean F_{ST} between sympatric species was high at all localities (range 0.70 to 0.95, mean = 0.86). We thus observed a lack of sympatric forms with 'intermediate' F_{ST} values between 0.30 and 0.70, representing a gap in the speciation continuum. To study this gap while accounting for non-independence of pairwise F_{ST} estimates, we estimated a phylogeny-based genealogical sorting index (GSI)[54]. Largely consistent with the F_{ST}-based results, we found strong bimodality in the distribution of GSI values for sympatric taxa, with a paucity of values intermediate between those characteristic of ecotypes and species (Fig. 5).

In contrast to sympatry, we found that conspecific populations in different localities (i.e., outside of sympatry) exhibit a wide range of differentiation, including levels intermediate between sympatric ecotypes and species (range of mean F_{ST} = 0.04 to 0.88, mean = 0.43, n = 579 pairwise comparisons; Fig. 5 for GSI). Specifically, such populations showed positive associations between mean F_{ST} and geographic distance (slope within all species > 0.30, all pp > 0.98, Bayesian Regression).

Analyses in ENTROPY[53] revealed little or no admixture between sympatric species, consistent with strong or complete RI (Fig. S1; Tables S2, S3). The documented gap between sympatric ecotypes and species thus likely reflects intraspecific gene flow (i.e., incomplete RI) that prevents maximal differentiation from forming or being maintained in sympatry. In principle, the gap could be due to rapid sympatric speciation. However, this is difficult theoretically[2,18]

1 and it does not match biogeographic patterns in Timema, where range overlap between
2 taxonomically recognised species is slight or absent[36]. Our results suggest that gene flow can
3 contribute to evolutionary gaps. Specifically, gene flow can make intermediate phases of
4 speciation difficult to observe because these phases occur rapidly (e.g., in reverse), rarely, or
5 restricted in space. In such cases, gaps are 'apparent' rather than real and extensive sampling is
6 required to observe intermediate states.
7
8 The evolution of complete RI
9
10 We have shown that maximal genomic differentiation in Timema is associated with complete
11 RI[13]. We thus studied the evolution of complete RI. We did so in the context of allopatric or
12 completely reproductively isolated species. Dynamics with gene flow could be different than
13 described below.
14
15 We quantified sexual isolation between Timema species from published data[45]. This revealed
16 some overlap within and between species, but greater sexual isolation on average between
17 species (Fig. 6). To study temporal dynamics of sexual isolation, we used divergence times
18 between species extracted from a Bayesian phylogenetic time-tree inferred using the GBS data
19 from our genus-wide survey, and dated with fossil-based secondary calibrations (Tables S11-
20 S14). This approach revealed that sexual isolation accumulates gradually through time until it
21 approximates completion (i.e., ~ 1, Fig. 6). Strong sexual isolation requires tens of millions of
22 years (Timema are univoltine with one generation per year). Morphological differentiation in
23 colour and other traits likely reduces the time to complete RI, by causing ecological
24 isolation[37,38]. However, morphological differentiation estimated here (n = 978) also evolves
25 gradually between species such that complete RI by sexual isolation plus ecological isolation
26 likely requires substantial time (Fig.6, Tables S11, S12). The long time frames required for
27 strong RI via the reproductive barriers measured here suggest that speciation in Timema
28 involves other barriers, such as genetic incompatibilities. Moreover, completion of RI could
29 involve long periods of geographic isolation. Future work on the most advanced stages of
30 Timema speciation is warranted.
31
32 Conclusions
33
34 We have shown that the transition from localised to genome-wide differentiation can be
35 observed despite gene flow, and may be aided by mate choice. Overall, our results accord well
36 with models of parapatric speciation[18], but do not support a strong role for the growth of a few
37 islands of differentiation, at least for early to intermediate phases of speciation. Details of the
38 evolution of strong RI in Timema remain unclear, but the existence of a wide range of
39 differentiation outside of sympatry facilitates future studies of many phases of speciation and
40 the role of coupling of differentiation across loci[3]. The myriad of effects reported here, and the
41 modesty of some of them, indicate that future work on the relative importance of each (rather
42 than merely its presence) is justified. Despite need for further work, our results show that
43 integrative studies do allow even complex speciation processes to begin to be understood.
44
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Author Information. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to RR (rudiger.riesch@rhul.ac.uk), ZG (zachariah.gompert@aggiemail.usu.edu), and PN (p.nosil@sheffield.ac.uk).

Figure 1. Conceptual overview and summary of genomic differentiation in Timema. (A) Genetic differentiation (red boxes) spreads to involve more of the genome as speciation progresses (adapted from[4]). Double-headed arrows represent gene flow between populations. Ticks above the horizontal line for Pop. (population) 1 represent genetic regions affected by divergent selection. The trajectory of increase in genomic differentiation can be affected by many factors, such as the genetic architecture of traits under selection, strength of selection, recombination rate variation, migration rate between populations, etc[1]. The top dotted line represents conditions where genome-wide differentiation evolves early during speciation. The bottom dotted line represents cases where genomic differentiation may be restricted to a few regions ('islands') for a substantial portion of the speciation process. (B) Summary of patterns of genomic differentiation in Timema. Divergent selection on colour-pattern loci is associated with localised differentiation, increased genome-wide differentiation is associated with CHCs, and the most pronounced levels of differentiation are associated with very low gene flow (i.e., due to complete reproductive isolation, RI, or strong spatial separation). Because genome-wide differentiation appears common in Timema, its trajectory may mirror the top dotted line in panel A.

Figure 2. Localised genetic differentiation (F_{ST}) in Timema cristinae. (A) Illustrations of Adenostoma and Ceanothus ecotypes of T. cristinae and their host plants. (B) Hidden Markov Model (HMM) results showing regions of accentuated F_{ST} (in red) relative to the genome-wide background (in grey). Single-nucleotide polymorphisms associated with colour-pattern map to LG8 and are found in regions of accentuated differentiation more than expected by chance. (C)

1 A steep cline in allele frequency at the colour-pattern locus, inferred from morph frequencies
2 (grey shaded areas are ± 95% credible intervals). (D) A HMM analysis of the within-
3 generation transplant experiment, showing regions of accentuated F_{ST} in red. The y-axis has
4 been corrected for minor variation in F_{ST} at the onset of the experiment, and thus represents
5 differentiation that evolved between the onset and completion of the experiment. (E) The
6 number of regions of accentuated differentiation per LG as a function of LG size, in the
7 transplant experiment (note the highest concentration on LG8). LG = linkage group.
8
9 **Figure 3. Cuticular hydrocarbons (CHCs) and genome-wide differentiation in Timema**
10 **cristinae.** (A) Time to copulation as a function of perfuming treatment. (B) Illustration of
11 representative methylated CHC profiles of females from two host-plant ecotypes (pA =
12 picoAmpere). (C) Differences between sexes and host ecotypes in CHCs (means ± 95%
13 confidence intervals (CIs)). A = Adenostoma, C = Ceanothus. (D) Tests as to whether male
14 (M, grey lines) or female (F, red lines) CHCs are associated with sexual isolation (SI) or mean
15 genome-wide differentiation (GD), after controlling for geographic distance. Shown are
16 posterior probability (pp) distributions for the effect size on each variable. (E) Percent variance
17 explained (PVE) by genotype in genome-wide association (GWA) mapping. Bars show
18 posterior medians and lines denote the 95% equal-tailed probability intervals. Shown in boxes
19 above each bar are r^2 values from cross-validation analyses (asterisks denote significance; $^{*}P <$
20 0.05, $^{**}P < 0.01$, $^{***}P < 0.001$). Abbreviations are % striped = percent of body area striped and
21 for methylated CHCs are as follows: fpenta = female pentacosanes, fhepta = female
22 heptacosanes, fnona = female nonacosanes, mpenta = male pentacosanes, mhepta = male
23 heptacosanes, mnona = male nonacosanes. (F) Linkage-group partitioning showing the number
24 of trait-associated SNPs as a function of linkage group (LG) size.
25
26 **Figure 4. Whole-genome analyses of genomic differentiation (F_{ST}) in Timema.** Hidden
27 Markov Model (HMM) results showing regions of accentuated differentiation (in red) relative
28 to the genome-wide background (in grey). Abbreviations by the species names are locality
29 codes and all taxon pairs are found on different host plants. Inset shows mean F_{ST} for regions
30 of background differentiation. LG = linkage group.
31
32 **Figure 5. A gap in genomic differentiation (mean genome-wide F_{ST}) for Timema taxa in**
33 **sympatry.** (A) A gap in genome-wide F_{ST} between conspecific host-plant-associated
34 populations and species within the same locality (i.e., 'sympatry'), estimated using genotyping-
35 by-sequencing data. (B) The gap using mean values per species and species pairs. (C)
36 Genealogical Sorting Index (GSI) analysis shows a paucity of intermediate values between
37 conspecific ecotypes and species (note that species level is restricted to species sympatric with
38 other species). (D) Time-calibrated phylogenetic tree of the relationships between the Timema
39 populations and species studied in our survey of 1505 individuals from 11 species in 47
40 geographic localities (= 57 tips in the tree). Bayesian Posterior Probabilities were >0.97 for all
41 nodes in the tree.
42
43 **Figure 6. Temporal dynamics of the evolution of sexual isolation and morphological**
44 **differentiation.** (A-C) Differences among populations and species in sexual isolation
45 (measured by the I_{PSI} index) and morphological differentiation. Dark red signifies overlapping
46 parts of the distributions shown. (D-F) Sexual isolation and morphological differentiation
47 between species against divergence time (Ma = million years; which in Timema is equal to
48 millions of generations). The regression line fitted using divergence times from the dated
49 molecular phylogeny is shown in black. 95% confidence intervals in grey shading were

1 obtained by fitting regression lines to the 2.5% and 97.5% quantiles of the distribution of
2 divergence times obtained from 1000 trees from the posterior distribution.
3
4
5 **Materials and Methods**
6
7 **Methods Summary.** We combined linkage mapping, phenotypic and experimental data,
8 genome-wide association (GWA) mapping, genotyping-by-sequencing (GBS) data, and whole-
9 genome re-sequence data from 1012 Timema individuals (160 genomes re-analysed from[13] and
10 852 new to this study, 473 of which originated from the transplant experiment and 379 from
11 natural populations of eight species). Table S15 provides an overview of the data used in this
12 study that was previously published, the data that are new, and the relation between the two.
13
14 For whole genomes, coverage is as follows: natural taxon pairs, mean coverage is ~1.1× per
15 individual and ~22.0x per population; transplant experiment ~1.4× per individual and ~139.4×
16 per experimental block. Coverage for GBS data was higher, as outlined below. In all cases, we
17 infer genotypes probabilistically, and thus account for genotype uncertainty (details below).
18 Such approaches are increasingly common in large-scale analyses in model systems, are not
19 reliant on 'calling' genotypes with certainty, and are suitable for robust inferences using low
20 coverage data across many individuals.[55,56]
21
22 Due to the size and complexity of our integrative data set, we provide the core methods below
23 in sufficient detail to evaluate our study. Further details concerning, e.g., read counts, sample
24 populations, and parameter settings, are contained in the Online Supplementary Materials
25 (OSM).
26
27 **Morph frequency cline.** We sampled T. cristinae at 33 collection sites in 1996 and again in
28 2001. We collected a total of 1598 individuals, and scored each as green-unstriped, green-
29 striped, green-intermediate, or melanistic (by CS in 1996 and by CS + PN in 2001). We first
30 considered just the green-striped and green-unstriped morphs, because these can be scored
31 unambiguously, and because the stripe is recessive such that green-striped morphs are
32 homozygous for the stripe allele and can be used to estimate the frequency of the major-effect
33 stripe allele[42]. We obtained estimates of the stripe allele frequency for each site by pooling data
34 across years (as results were similar across years) and by assuming that all striped individuals
35 were homozygous for the stripe allele and Hardy-Weinberg equilibrium. We fit a 6-parameter
36 cline model for the stripe allele frequencies[57] using the R 3.2.3 package hzar 0.2-5[58]. We
37 inferred cline parameters in a Bayesian framework using Markov chain Monte Carlo (2 million
38 iterations with a 1-million iteration burn-in). To assess the robustness of our results, we
39 repeated this analysis including individuals scored as intermediate, assuming they were green-
40 striped morphs, and assuming they were green-unstriped morphs. We observed a qualitatively
41 similar conclusion of a steep cline in all analyses, although quantitative details varied among
42 the analyses (Fig. S3).
43
44 **Whole-genome analyses of published T. cristinae genomes.** We conducted novel analyses of
45 larger-scale heterogeneity in genetic differentiation between the Adenostoma and Ceanothus
46 ecotypes of T. cristinae. The analyses based on 20-kb windows thus differ from previous work
47 that analysed fine-scale differentiation of SNPs for these same ecotypes. We used a Hidden
48 Markov Model (HMM) to identify contiguous genomic regions with accentuated
49 differentiation between each of four previously studied T. cristinae ecotype pairs (HVA ×
50 HVC, MR1A × MR1C, R12A × R12C, and LA × PRC). These data were described in[13] and

1 include 160 whole genome sequences. We first calculated F_{ST} for non-overlapping 20-kb
2 windows as $F_{ST} = (\pi_t - \pi_w)/ \pi_t$, where π_w is the mean nucleotide diversity within ecotypes and
3 π_t is the nucleotide diversity for both ecotypes combined. Note that we calculated our estimate
4 as a ratio of means across sites (rather than a mean of ratios) as suggested by[59]. We then fit a
5 HMM with two discrete states for the logit transformed F_{ST} estimates for each ecotype pair,
6 assuming logit F_{ST} was normally distributed. We defined a background differentiation state
7 with a mean and standard deviation that matched the empirical mean and standard deviation,
8 and an accentuated differentiation state with the same standard deviation but a mean set to the
9 90th empirical quantile of the F_{ST} distribution. We estimated the transition matrix between
10 states using the Baum-Welch algorithm, and we used the Viterbi algorithm to predict the most
11 likely sequence of hidden states from the data and estimated parameters[60]. We used the R 3.0.2
12 package HiddenMarkov 1.7.0 to fit these models[61,62] but modified the code to use fixed values
13 for state means and standard deviations (this allowed us to explicitly test of islands of
14 accentuated differentiation). We defined HMM regions of accentuated differentiation as the
15 contiguous set of 20-kb windows showing a high differentiation state within a linkage group
16 (but potentially spanning multiple scaffolds).
17
18 **Co-localisation of stripe-associated SNPs and HMM regions of accentuated**
19 **differentiation.** We applied this analysis to the four pairs of Ceanothus and Adenostoma
20 ecotypes of T. cristinae[13], which are known to be subject to divergent selection on colour-
21 pattern. We used a permutation test to ask whether stripe-associated SNPs from our GWA
22 mapping (described below) occurred in high HMM regions across the four ecotype pairs more
23 often than expected by chance. However, as only two pairs had high HMM regions (on LG8
24 for R12A × R12C and LGs 1 and 8 for LA × PRC), this is really a test of whether stripe-
25 associated SNPs were in high HMM regions more than expected by chance for these two pairs.
26 We focused on SNPs with posterior inclusion probabilities for stripe that were greater than 0.1.
27 Such SNPs occurred in seven unique 20-kb windows. Across the four pairs, the windows with
28 stripe-associated SNPs were also high HMM windows 20% of the time. Randomisation of high
29 HMM regions (10,000 randomisations, with the size of HMM regions kept constant) indicated
30 that high HMM regions and trait-associated SNP regions overlapped more than expected by
31 chance (null expectation = 1.7%, $P = 0.0033$). We obtained similar results when considering
32 stripe-associated SNPs with posterior inclusions probabilities greater than 0.05 (17 unique 20-
33 kb windows, observed overlap = 14%, null expectation = 1.7%, $P = 0.0003$).
34
35 **Whole-genome transplant and sequence experiment.** As the procedures for implementing
36 this experiment have been previously described[63], we provide here only a brief overview. We
37 collected and transplanted 500 T. cristinae from an area dominated by Adenostoma (population
38 FHA) onto either an individual of their native host plant (Adenostoma) or the alternative host
39 plant (Ceanothus). As previously described[63], there is little to no dispersal in such experimental
40 settings, including the experiment analysed here. After eight days, we recaptured surviving
41 insects. Following previously published protocols[13], we then extracted DNA, prepared
42 individually-barcoded sequencing libraries, and conducted whole-genome re-sequencing of the
43 500 insects. We successfully obtained data from 473 individuals, which we analysed further.
44 We aligned the paired-end sequences to the T. cristinae reference genome using the BWA-
45 MEM algorithm in BWA 0.7.5a-r405 [64]. We then identified variant nucleotides using the
46 UnifiedGenotyper in GATK 3.1 (ignoring scaffolds not assigned to LGs) and estimated
47 genotypes using an empirical Bayesian approach, as in past work[63].
48
49 We quantified genetic differentiation between survivors from the two host plant treatments by
50 calculating F_{ST} for 20-kb windows, as described in the previous section. We likewise

1 calculated F_{ST} at the onset of the experiment, verifying that genetic differentiation at the start
2 was low to non-existent. We then fit the same HMM described in the preceding section to
3 delineate accentuated regions of genetic differentiation between survivors on Adenostoma
4 versus Ceanothus, controlling for minor variation in genetic differentiation at the onset of the
5 experiment by subtracting initial F_{ST} from F_{ST} between the survivors. We conducted a
6 randomisation test (1000 permutations of HMM window states) to determine whether HMM
7 windows assigned to the high differentiation state occurred on LG8 more than expected by
8 chance.
9
10 **Quantifying dorsal colour-pattern (% body area striped).** We recorded digital images of
11 873 adult T. cristinae (539 males and 334 females) using previously described methods[42]; 592
12 of these images (395 males and 197 females) stem from a previous study that considered a
13 single population on Adenostoma (FHA, i.e., one ecotype in one locality) and that used the
14 images to quantify and map colour-pattern (% striped)[42]. Here, we estimated % striped for the
15 full set of photos, including eight populations on Ceanothus and 10 on Adenostoma. These data
16 were collected to facilitate tests on the effect of colour-pattern on genomic differentiation
17 among populations, but GWA was restricted to individuals from the large sample in FHA. We
18 estimated % striped by dividing the area of the stripe by the total dorsal body area, each
19 estimated using the "polygon selection tool" in ImageJ, as previously described[42].
20
21 **Cuticular hydrocarbon (CHC) variation.** We sampled 20 populations of T. cristinae (eight
22 on Ceanothus and 12 on Adenostoma) for a total of 915 insects (559 males and 356 females;
23 Table S6). As above, a subset of these stem from the FHA population reported in[42], but
24 ecotype differences in CHCs or the genetic basis of CHCs were not examined in this previous
25 study. We cold-euthanized live insects, and subsequently submerged them in separate vials
26 with 1 ml of HPLC-grade hexane for 10 minutes to extract CHCs from their body surface.
27 Using a 6890 Hewlett Packard (now Agilent) gas chromatograph (GC), we quantified 26
28 different mono- and di-methylated CHCs for each insect: eight pentacosanes, eight
29 heptacosanes, and 10 nonacosanes. As is standard practice in studies of CHCs[45], we analysed
30 their proportional rather than absolute abundance; this allowed us to reduce experimental error
31 and to remove individual differences stemming from variation in insect body size[65,66]. We
32 calculated CHC proportions by dividing the amount of each CHC in a given sample by the sum
33 of all quantified CHCs in that sample. We then transformed these CHC proportions using log-
34 contrasts[65,67] to remove the non-independence among analysed variables. We calculated log-
35 contrasts by dividing the value for each CHC by the value of the CHC 5-methylheptacosane
36 (5Me27), and then taking the $\log_{10}$ of these new variables, resulting in 25 log-contrast
37 transformed values for every insect. We found all 25 CHC-measurements to be highly
38 repeatable, and the results obtained by dividing by values of other CHCs to be similar (OSM).
39 To further reduce data dimensionality and to account for multicollinearity, we conducted a
40 principal components analysis (on a covariance matrix with promax rotation) and retained
41 principal component (PC) axes with an eigenvalue larger than the mean eigenvalue as variables
42 in a multivariate analyses of variance (MANOVA) to test for effects due to ‘sex’, ‘host plant’,
43 and the interaction of ‘sex-by-host plant’ (12 populations on Adenostoma and eight on
44 Ceanothus).
45
46 **Genotyping-by-sequencing (GBS) and genome-wide association (GWA) mapping.** We
47 obtained genotypes for mapping with 592 T. cristinae from the FHA population using the
48 sequencing reads from these insects previously published[42]. This previous study mapped
49 colour-pattern (% striped) but not the other traits considered here. We used the software
50 GEMMA 0.94[48] to implement Bayesian sparse linear mixed models (BSLMMs) that estimate the

1 genetic architecture of traits while also considering relatedness of individuals within the
2 sample. BSLMMs in GEMMA provide estimates of the proportion of phenotypic variation that
3 can be explained by the combined effects of polygenic (infinitesimal effect) and measurable
4 (modest to larger) effect SNPs. We thus estimated three hyper-parameters for each trait: (i) the
5 total proportion of phenotypic variance explained (PVE) by genotype (i.e., estimated
6 heritability), (ii) the proportion of the genetically explained phenotypic variation (i.e., PVE)
7 that is due to the effects of measurable-effect SNPs (PGE), and (iii) the number of measurable-
8 effect SNPs (n-SNP). GEMMA also provides posterior inclusion probabilities (PIPs, also called
9 γ parameter) for each SNP that reflect the fraction of Markov-Chain-Monte-Carlo (MCMC)
10 iterations of the BSLMM for which a given SNP had a measurable effect on phenotypic
11 variation (i.e., this reflects the weight of evidence that individual SNPs are associated with the
12 trait of interest).
13
14 We estimated the above-mentioned hyper-parameters and PIP values for the following traits:
15 (i) % striped, (ii) the proportion of methylated pentacosanes, heptacosanes, and nonacosanes in
16 females (fpenta, fhepta, and fnona, respectively), and (iii) the proportion of methylated
17 pentacosanes, heptacosanes, and nonacosanes in males (mpenta, mhepta, and mnona,
18 respectively). We tested for an association between the number of trait-associated SNPs per
19 LG and the LG size; a strong positive correlation is predicted for polygenic traits[47]. Finally, we
20 performed cross-validation (i.e., genomic prediction) analyses to test the predictive power of
21 our GWA mapping[68].
22
23 **Perfuming trials with no-choice copulation experiments.** We conducted 24 no-choice
24 copulation trials (eight trials each with 'conspecific native population perfume', 'heterospecific
25 perfume', or 'no perfume') between one male and one female T. cristinae from FHA (males
26 choose mates in Timema)[49]. Each individual perfume consisted of CHCs that we extracted
27 from six cold-euthanized females, and that we gently transferred to the live female in each
28 trial. No-choice copulation trials were based on previously published protocols[69]. For each
29 trial, we kept one male and one female T. cristinae in a 10-cm Petri dish for 4 h, and we scored
30 the latency to copulate (i.e., minutes until copulation)[49]. We conducted perfuming trials during
31 the same time (8:45 am – 12:45 pm) on different days, but always ran the same number of
32 'conspecific' and 'heterospecific' perfuming trials simultaneously. We analysed the latency to
33 copulate by means of a Kaplan-Meyer analysis in IBM SPSS Statistics 21.
34
35 **Tests for effects of colour-pattern and CHCs on sexual isolation and genome wide F_{ST}.**
36 These analyses focused on seven T. cristinae populations previously studied for sexual
37 isolation, for which we also obtained data on colour-pattern, CHCs, and F_{ST} (n = 21 pairwise
38 comparisons). We estimated the strength of sexual isolation between populations by
39 calculating the I_{PSI} index (theoretical range –1 to +1, where –1 = complete disassortative
40 mating, 0 = random mating, +1 = complete sexual isolation; all our empirical values were
41 positive)[70]. Specifically, we calculated pairwise I_{PSI}-scores based on mating propensity derived
42 from no-choice mating trials published in a previous study[43]. We estimated CHC differences
43 between populations, as follows: we first conducted PC analyses separate for each sex (on a
44 covariance matrix with promax rotation) on CHC data from these seven populations. We
45 retained PC axes with an eigenvalue larger than the mean eigenvalue to calculate sex-specific
46 pairwise Euclidian CHC distances between populations. We estimated population
47 differentiation in colour-pattern using data on morph frequencies (green-striped versus green-
48 unstriped) collected between 2000 and 2008 (population, % striped morph, sample size: PC,
49 18, 505; HVA, 85, 1383; MA, 82, 310; LA, 86, 654; OUTA, 49, 631; PRC, 1, 1261; OGC,
50 7168).

1
2 To obtain F_{ST} estimates, we combined new GBS data for 325 samples from 19 T. cristinae
3 populations with 17 randomly chosen samples (10 males and 7 females) from the FHA
4 mapping population, resulting in sequences from 342 individuals spanning 20 populations (5 -
5 20 individuals per population, mean = 17) for population genetic analyses of genetic
6 differentiation. We mapped reads to the reference genome with BOWTIE2 2.2.3 and called
7 variants with SAMTOOLS 0.1.19 mpileup and BCFTOOLS 0.1.19 using the full prior and
8 requiring the probability of the data being homozygous for the reference allele to be less than
9 0.01. We estimated genome-wide Hudson's F_{ST}[71,72] for all 190 population pairs using allele
10 frequencies estimated from genotype probabilities obtained as in[13]. We retained 613,261 bi-
11 allelic SNPs with mean coverage depth per SNP per individual ~5× (per SNP average ranging
12 from 2.2 to 28.7; per individual average ranging from 1.0 to 10.3).
13
14 We estimated genome-wide Hudson's F_{ST}[71,72] for all 190 population pairs as $F_{ST} = 1 - Hw/Hb$.
15 Hw is the mean number of differences among sequences from the same population, and Hb the
16 mean number of differences among sequences from different populations, averaged over loci.
17 We calculated Hw and Hb for each locus from population allele frequencies estimated using
18 genotype probabilities obtained with SAMTOOLS and BCFTOOLS[73], as in[13]. For each population
19 pair, we excluded loci with a MAF less than 0.05, or where less than 50% of individuals were
20 covered.
21
22 We used these data for subsequent tests of how colour-pattern and CHCs affect sexual isolation
23 and mean F_{ST}. As reported in the main text, we fit Bayesian linear mixed models to test for
24 effects of population differentiation in these traits on sexual isolation and mean F_{ST} while
25 accounting for geographic distances among populations and the correlated error structure of
26 pairwise distance data[53,74]. We did this using either sexual isolation or logit-transformed mean
27 F_{ST} as the response variable (in the former case we also conducted analyses replacing
28 geographic distance with mean F_{ST} as the covariate being accounted for). Linear models
29 included population-specific random effects, geographic distances, and one of the three
30 following variables as predictors of sexual isolation or F_{ST}: (i) colour-pattern distances (%
31 difference between populations in striped individuals), (ii) male CHC distances, or (iii) female
32 CHC distances. We centred and standardized covariates prior to analyses. We specified
33 uninformative priors for the regression coefficients (normal priors with $\mu = 0$, $\sigma^2 = 1000$) and
34 for the gamma ($\alpha = 1$, $\beta = 0.01$) hyper-priors on the precision (inverse variance) for the random
35 effects[53]. We ran three independent MCMC chains each with 5000 iterations, a 1000 iteration
36 burn-in, and a thinning interval of five for each model. We then calculated the posterior
37 probability that the standardized partial regression coefficient for colour-pattern, male CHC, or
38 female CHC distance was greater than zero (this is valid as the effect of having pairwise
39 observations is accounted for by the population random effects)[53,74].
40
41 **Whole-genome re-sequencing of 10 population pairs spanning eight species.** Following
42 previously published protocols[13], we sequenced and further analysed an additional 379 Timema
43 genomes (these are a subset of the 1505 described below for which we obtained genotyping-
44 by-sequencing data). We aligned the paired-end sequences to the T. cristinae reference genome
45 using the BWA-MEM algorithm in BWA 0.7.5a-r405 and identified SNPs using the
46 UnifiedGenotyper in GATK. We used an expectation-maximization (EM) algorithm to obtain
47 maximum-likelihood-allele-frequency estimates for each of the 20 populations (10 'parapatric'
48 population pairs) for each of 5.07 million identified SNPs. We then used these maximum-
49 likelihood-allele-frequencies to calculate sequence-based estimates of F_{ST} between each of the
50 10 co-occurring taxon pairs, as described above for ecotypes of T. cristinae. Additionally, we

1 determined Nei's measure of absolute divergence (D_{XY})[75] for each 20-kb window for the two
2 hetero-specific population pairs (LP and SM). We used Approximate Bayesian Computation
3 (ABC) to estimate migration rates between these 'parapatric' taxa based on a non-equilibrium
4 Wright-Fisher model with gene flow, and also provide estimates under an island equilibrium
5 model[76]. We used the Hidden Markov Model[40] approach employed in T. cristinae to assign
6 each of the 20-kb windows into groups of background or accentuated (i.e., 'high') levels of
7 differentiation. Finally, we quantified minor allele frequencies (MAFs) for HMM regions of
8 accentuated differentiation (for the taxon pairs where such regions were detected), and
9 compared them to MAFs for the genomic background. We did so for the previously published
10 ecotype pairs[13], and the 10 pairs with new whole-genome data (Table S5).
11
12 **Genotyping-by-sequencing (GBS) and stages of speciation.** We sampled 47 widely
13 distributed geographic localities across California for Timema, with the over-arching goal of
14 sampling the greatest possible diversity of hosts, localities, and sexual Timema species. In
15 total, we collected 1545 individuals of 12 Timema species (one sample was from an asexual
16 species) from 13 host plant genera. The data set includes all the Californian sexual species of
17 Timema (the others are found outside California). We extracted DNA and prepared libraries for
18 GBS sequencing of all these individuals, as in previous work[39].
19
20 We aligned reads to the T. cristinae reference genome[13] using BOWTIE2 2.1.0[77]. Quality control
21 filtering resulted in a dataset of 1505 individuals from 11 species that we used for all
22 downstream analyses. Variants were called using SAMTOOLS mpileup and BCFTOOLS using the
23 full prior, requiring the probability of the data to be less than 0.5 under the null hypothesis that
24 all samples were homozygous for the reference allele to call a variant. We ignored insertion
25 and deletion polymorphisms. For each population and variant, we inferred maximum-
26 likelihood allele frequencies from the genotype likelihoods by means of the iterative soft
27 expectation-maximization algorithm (EM) described in[73], and measured genome-wide genetic
28 differentiation between pairs of populations using the Hudson's F_{ST}[72].
29
30 For conspecific populations found in different geographic localities we used a Bayesian
31 hierarchical regression model to quantify the association between log geographic distance and
32 logit F_{ST}. Slope and intercept terms were modelled hierarchically and allowed to vary by
33 species. Non-informative priors were placed on the overall (across species) intercept and slope
34 coefficients (Normal(mu = 0, tau = 1e-6) for means, and gamma (alpha = 0.01, beta = 0.01) for
35 all precision terms. Parameters were inferred using MCMC via the rjags interface with R. We
36 ran three chains, each with a 20,000 iteration burn-in, 50,000 sampling iterations and a
37 thinning interval of 10.
38
39 We estimated genetic structure and potential admixture using a hierarchical Bayesian model
40 that jointly estimates genotypes and admixture proportions as implemented in the program
41 ENTROPY 1.2b [53]. This model is similar to the popular STRUCTURE algorithm[78] but accounts for
42 sequencing errors and genotype uncertainties inherent to next-generation sequencing methods
43 in a way comparable to other approaches[79]. We estimated parameters for a model with K=2
44 population clusters for every pair of populations found at the same geographic locality but
45 belonging to different species, and K=number-of-host-plants clusters for conspecific
46 populations found at the same locality. Moreover, we used the Deviance Information Criterion
47 (DIC) to evaluate if the models fitted better than K=1.
48
49 **Maximum-likelihood phylogenetic inference and genealogical sorting index (GSI).** We
50 inferred 1000 maximum-likelihood bootstrap trees using the rapid heuristic algorithm

implemented in RAxML 8.2.9[80,81]. We used a curated dataset of 19,556 single nucleotide variants (SNVs) for 1505 individuals, which we partitioned by linkage group. We calculated the genealogical sorting index (GSI) using the R package genealogicalSorting 0.92[54]. GSI is a statistic that measures the degree of exclusive ancestry of groups of individuals in a tree. It ranges from 0 when all the nodes of the tree are required to unite the group, to 1 when a group is genealogically exclusive (i.e. individuals are united by the minimum possible number of nodes). For each bootstrap tree, we calculated GSI values for the 166 groups with at least 2 individuals delimited by species (11), species and locality (56), and species, locality, and host (98). We plotted the joint distribution of GSI values from all bootstrap trees for sympatric species (5), geographic localities within species (37), and conspecific host ecotypes within localities (90) (Fig. 5C). Bootstrap trees and tables with GSI values are deposited in Dryad.

Estimation of sexual isolation and morphological differentiation between species. We estimated sexual isolation between species by calculating the I_{PSI} index on previously published mating trial data within and between species[45]. We excluded the data from T. boharti due to uncertain species ID, but including them does not alter our conclusion. To measure morphological differentiation within and among species, we measured morphological traits of 978 adult individuals from different Timema species (Tables S11, S12). We captured specimens by sweep netting their host plants in localities that broadly overlap with those used in our genetic survey. We photographed specimens with a digital Canon EOS 70D camera equipped with a macro lens (Canon EF 100mm f/2.8L Macro IS USM) and two external flashes (Yongnuo YN560-II speedlights). We took the images with the camera set on manual, an aperture of f/14, a shutter speed of 1/250 s, and flashes adjusted to 1/4 power in S2 mode in an output angle corresponding to 24-mm focal length on full frame (~84° diagonal). To avoid strong shadows and create an even, soft lighting, we diffused both flashes with LumiQuest SoftBox LTp softboxes, following the manufacturer's instructions. With these flash adjustments, we were able to standardise the light reducing external luminosity interference. In addition to Timema specimens, the pictures included a ruler and a standard colour chip (Colorgauge Micro, Image Science Associates LLC, Williamson, NY, USA). We photographed each insect at least twice, in positions that varied perpendicularly to capture the body colour without traces of gleam or shade. We linearized and corrected each picture for the white balance, adjusting the Temperature and the Tint based on the values obtained from the colour chip neutral grey colour (target #10), using ADOBE PHOTOSHOP LIGHTROOM 5.7 software (Adobe Systems Software Ireland Ltd). Only minor corrections were necessary, as the measurements did not vary appreciably among pictures. We adjusted pictures for the Temperature to 5950 and for the Tint to +2, and exported them as TIFF files.

From the standardized images we collected phenotypic measurements using the software IMAGE J 1.4.8[82]. We extracted the following size measurements: (i) body length (BL, from the tip of the head to the base of the abdomen, not including external genitalia), (ii) body width (BW, the widest point of the second thoracic segment), and (iii) head width (HW, the distance between the eyes). We scaled the pictures using the ruler as reference, thus being able to convert all linear measurements from units of pixels into centimetres. To quantify variation in colour, we recorded mean RGB (Red, Green, Blue) values using the polygon section tool and colour histogram plugin in ImageJ. We took the colour measurements on the lateral and dorsal margin of the second thoracic and fourth abdominal segments. We obtained the mean between the two measurements done in the lateral margin and between the two in the dorsal part. We then converted these raw RGB values to variables representing two colour channels and one luminance channel, as previously suggested[83]. We calculated a red-green (RG) colour channel using the relationship (R-G)/(R+G), a green-blue (GB) colour channel as (G-B)/(G+B), and a

1 luminance (L) (i.e., brightness) channel as (R+G+B). While this method of measuring colour
2 does not account for how colour is sensed by a potential receiver (e.g., conspecific or predator),
3 it does represent an unbiased quantification of colour that is useful in a comparative context.
4
5 We thus describe morphology based on size (i.e., BL, BW, HW) and on colour channels, with
6 values for lateral red-green (latRG), lateral green-blue (latGB), lateral luminance (latL), dorsal
7 red-green (dorRG), dorsal green-blue (dorGB), and dorsal luminance (dorL). Following trait
8 measurements, we performed a principal component analysis (PCA) using all measured traits,
9 extracting the scaled score of the first four axes for each individual. We conducted separate
10 PCA analyses for each sex, given notable sexual dimorphism in the morphology. The first four
11 axes account for 87 and 83% of the variation in males and females, respectively (Table S12).
12 We then estimated morphological distances using pairwise Euclidean distance values between
13 different species and among populations within species, following[84].
14
15 **Phylogenetics and molecular dating.** On account of the absence of Timema fossils and the
16 poor fossil record of stick insects, we used secondary calibrations derived from a time-
17 calibrated tree of insects (Tables S13-14). To infer such a tree, we retrieved from GenBank
18 sequences of nine molecular markers (four mitochondrial genes and five nuclear genes) for 41
19 genera belonging to 13 orders, placing particular emphasis on ensuring a good representation
20 of stick insects and including the three main clades of Timema: Northern, Southern and Santa
21 Barbara. For divergence time estimation, we chose six calibrations for phylogenetically well-
22 supported groups based on robust fossil data (OSM for details; Table S13). We carried out
23 Bayesian phylogenetic inference with BEAST 2.1.3[85,86], which allows co-inference of tree
24 topology and divergence times using a relaxed molecular clock and incorporating uncertainty
25 in calibrations as priors in the form of statistical distributions. Subsequently, we used the
26 divergence time posterior distributions for the root of Timema (split between the Northern +
27 Santa Barbara clades and the Southern clade) and the split between the Northern clade and the
28 Santa Barbara clade for calibrating the tree of Timema populations based on GBS data (Table
29 S14). We inferred this tree using BEAST with the same curated dataset of 19,556 SNVs used for
30 the inference of maximum-likelihood bootstrap trees, but pooled by species and locality (for a
31 total of 57 populations). We partitioned by linkage group and incorporated secondary
32 calibrations as priors in the form of Γ distributions. Details concerning GenBank sequences,
33 multiple alignments, and phylogenetic trees are deposited in Dryad.
34
35 **All References in main text and methods**
36

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