









FROM THE COVER

Long-term balancing selection on chromosomal variants associated with crypsis in a stick insect

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Abstract

How polymorphisms are maintained within populations over long periods of time remains debated, because genetic drift and various forms of selection are expected to reduce variation. Here, we study the genetic architecture and maintenance of phenotypic morphs that confer crypsis in *Timema cristinae* stick insects, combining phenotypic information and genotyping-by-sequencing data from 1,360 samples across 21 populations. We find two highly divergent chromosomal variants that span megabases of sequence and are associated with colour polymorphism. We show that these variants exhibit strongly reduced effective recombination, are geographically widespread and probably diverged millions of generations ago. We detect heterokaryotype excess and signs of balancing selection acting on these variants through the species' history. A third chromosomal variant in the same genomic region likely evolved more recently from one of the two colour variants and is associated with dorsal pattern polymorphism. Our results suggest that large-scale genetic variation associated with crypsis has been maintained for long periods of time by potentially complex processes of balancing selection.

KEYWORDS

adaptation, chromosomal inversion, colour polymorphism, divergence, gene flow, heterozygote advantage

1 | INTRODUCTION

Crypsis is a widespread trait that reduces the risk of prey or predators from becoming initially detected when in plain sight, for example through background matching (Stevens & Merilaita, 2009). It is a central element in prey–predator interactions, and its selective advantage can be substantial and involve reduced metabolic costs and higher survival probability. However, much remains unknown about the details of the genetic basis of crypsis, and the evolutionary

processes involved in its origin and maintenance (Skelhorn & Rowe, 2016; Stevens & Merilaita, 2009). Here, we study the genetic basis and evolutionary processes that maintain different cryptic morphs within populations of a stick insect.

Although some species evolved only a single, highly optimized cryptic form, others are polymorphic. Novel morphs might recurrently develop but be transient through their subsequent replacement, for example if predators initially avoid unfamiliar morphs (predator wariness; Mappes, Marples, & Endler, 2005).

Polymorphisms might also be maintained by gene flow–selection balance (e.g., Hoekstra, Drumm, & Nachman, 2004; King & Lawson, 1995), negative assortative mating (e.g., Hedrick, Smith, & Stahler, 2016; Tuttle et al., 2016), or various mechanisms of balancing selection (Hedrick, Ginevan, & Ewing, 1976). Balancing selection can result from: (i) variable microhabitats that can induce spatially or temporally varying selection (Charlesworth & Charlesworth, 2010), (ii) frequency-dependent selection, for example, based on predator behaviour (apostatic selection; Allen, 1988; Bond & Kamil, 1998; Clarke, 1969) or (iii) heterozygote advantage. Despite this, changing selection pressures, allele turnover or genetic drift in finite populations are expected to eventually remove existing variants (Charlesworth, 2006; Charlesworth & Charlesworth, 2010). Consequently, balancing selection is often regarded a common, albeit predominantly short-term, mechanism for maintaining variation (Asthana, Schmidt, & Sunyaev, 2005; Charlesworth, 2006; Fijarczyk & Babik, 2015).

The nature of selection on colour polymorphisms can also affect their genetic architecture. For example, heterozygotes that exhibit intermediate trait values can be selected against if selection is divergent between discrete environments. Accordingly, many polymorphic species show dominant trait expression for colour patterns, often realized as dominance hierarchies in cases with more than two morphs (Clarke & Sheppard, 1972; Johannesson & Butlin, 2017; Joron et al., 2011). Given the potentially high selective pressure against maladapted colour morphs, finely tuned genetic architectures that result in strongly dominant trait expression can evolve, with heterozygotes being phenotypically similar or even identical to one homozygote (Le Poul et al., 2014). Alternatively, phenotypically different heterozygotes might not be selected against, for example, if intermediate niches exist or if effectively no intermediates occur because one of the homozygotes is lethal (Hedrick, 2012; Kuepper et al., 2016; Le Poul et al., 2014; Tuttle et al., 2016).

If more than one locus is required to generate alternative morphs and if recombinant phenotypes are selected against, genetic architectures that keep multiple adaptive alleles in linkage disequilibrium (LD) can evolve (Charlesworth, 2016; Charlesworth & Charlesworth, 1975; Kirkpatrick & Barrett, 2015; Yeaman, 2013). This can for instance be achieved by tight physical linkage, genetic modifiers of recombination, or structural changes such as chromosomal rearrangements. Chromosomal rearrangements have the advantage that they reduce recombination only in heterokaryotypes, thus facilitating purging of deleterious mutations through normal recombination in homokaryotypes (Kirkpatrick, 2010; Otto & Lenormand, 2002). However, chromosomal rearrangements can also reduce fitness in heterokaryotypes (Faria & Navarro, 2010; Kirkpatrick & Barton, 2006; Rieseberg, 2001), a situation that will act against the maintenance of rearrangement polymorphisms within populations.

Such selective processes acting on colour polymorphisms and the genetic architecture of the traits involved have been investigated in a variety of organisms, providing evidence consistent with the wide

range of ecological and genetic outcomes described above (e.g., Cook, 1998; van't Hof et al., 2016; Joron et al., 2011; Kuepper et al., 2016; Kunte et al., 2014; Lamichhane et al., 2016; Nachman, Hoekstra, & D'Agostino, 2003; Richards et al., 2013; Tuttle et al., 2016). However, less is known about the extent to which cryptic polymorphisms differ from the frequently studied colourful outcomes of sexual selection or mimicry (Stevens & Merilaita, 2009). Crypsis differs from mimicry as morph frequencies are independent of the population dynamics or evolution of a model species (Endler, 1981), and some morphs might become fixed by directional selection if they go undetected by predators (Bond & Kamil, 1998). Thus, processes other than apostatic selection or predator wariness might drive the maintenance of variation in species exhibiting cryptic phenotypes. In addition, it remains unclear how often cryptic polymorphisms are maintained within populations over long periods of time by balancing selection (Gray & McKinnon, 2007), versus being an ephemeral outcome of environmental changes (van't Hof et al., 2016) or a balance between gene flow and selection (e.g., Hoekstra et al., 2004; King & Lawson, 1995).

Here, we address these unresolved issues in understanding the evolution and maintenance of cryptic colour morphs by studying populations of the stick insect *Timema cristinae*. This species has three colour and colour–pattern morphs that are adapted to different microhabitats (details below). Combining genotyping-by-sequencing (GBS) and phenotypic data from hundreds of samples across 21 populations, we investigated the maintenance and genetic architecture of this polymorphism.

1.1 | Study system

The genus *Timema* comprises 21 described species of herbivorous stick insects in southwestern North America (Law & Crespi, 2002; Nosil, Crespi, & Sandoval, 2002; Sandoval, Carmean, & Crespi, 1998; Vickery & Sandoval, 2001). All *Timema* are wingless and rely on crypsis as protection against avian predators while resting on their host plants (Sandoval, 1994a). Their body coloration approximate colours of stems, fruits, needles or leaves of their hosts, and most species exhibit colour or colour–pattern polymorphisms (Comeault, Carvalho, Dennis, Soria-Carrasco, & Nosil, 2016; Comeault et al., 2015; Crespi & Sandoval, 2000; Sandoval et al., 1998). In two species (*T. cristinae* and *T. podura*), variation in colour or colour–pattern has been experimentally linked to fitness variation in the face of visual predation, supporting the adaptive nature of the polymorphisms and their role in crypsis (Nosil, 2004; Nosil & Crespi, 2006; Sandoval, 1994a,b; Sandoval & Nosil, 2005).

We focus here on *T. cristinae*, which is endemic to chaparral vegetation in a mountainous region surrounding Santa Barbara, California. *T. cristinae* is polymorphic for two distinct body colour morphs found within populations: a common green morph matching coloration of leaves, and a rarer melanistic (i.e., dark grey or red) morph approximating coloration of stems or fruits of their host plants, or soil (Figure 1a; Comeault et al., 2015; Sandoval, 1994a,b). These colour morphs will be referred to as “green” and “melanistic” hereafter.

Compared to green morphs, melanistic *T. cristinae* are more cryptic to avian predators on the woody tissue of their host plants but less so on leaves, and show higher resistance to fungal infections (Comeault et al., 2015).

Timema cristinae primarily uses *Adenostoma fasciculatum* and *Ceanothus spinosus* as host plants. As an adaptation to the differently shaped and coloured leaves of these host species, the green morph exhibits an additional polymorphism for the presence or absence of a white longitudinal dorsal stripe ("green-striped" and "green-unstriped" pattern morphs hereafter). The stripe visually divides the body into two slim parts and increases survival on the narrow needle-like leaves of *Adenostoma*. In contrast, the white stripe is conspicuous on the broad leaves of *Ceanothus* and reduces survival on this host (Nosil, 2004; Nosil & Crespi, 2006; Sandoval, 1994a; Sandoval et al., 1998).

Pattern morph frequencies vary across the landscape according to gene flow–selection balance between the often patchily distributed host plants (Sandoval, 1994b). Thus, the green-striped morph tends to be more common on *Adenostoma* and the green-unstriped morph more common on *Ceanothus* (Nosil et al., 2002; Sandoval, 1994a,b). By contrast, the melanistic morph, which does not express the stripe, occurs at ~11% within populations of either host plant and, although slightly more common in hot and dry climates, does not vary substantially in frequency across the landscape (Comeault et al., 2015). Thus, intrapopulation polymorphism of colour morphs is likely not strongly driven by gene flow between populations differing in morph frequency.

Previous studies suggest that the genetic architecture for either colour or pattern involves one or a few loci of large effect, and that colour and pattern loci are distinct but physically linked. These studies further showed that the green variant is fully dominant to melanistic, while the stripe is partially recessive (Comeault et al., 2015; Sandoval, 1994a,b). Thus, intermediates for colour do not exist, but incompletely or faintly green-striped *T. cristinae* are occasionally observed in the field (Sandoval, 1994a), reflecting the imperfect dominance for pattern (Comeault et al., 2015) or recombination among multiple loci controlling this trait.

Despite this background, numerous fundamental aspects of the evolution of these polymorphisms remain unresolved, which we investigated here. For example, whether different cryptic morphs have existed over extended periods of time is unknown, as is the potential contribution of different mechanisms of balancing selection or negative assortative mating in maintaining variation. It is also unknown whether the genetic architecture of cryptic traits involves reduced recombination between potentially many loci or is more aligned with a single locus. Our results show that the colour polymorphism in *T. cristinae* is not recent and involves a large genomic region under balancing selection that almost completely lacks genetic exchange between divergent variants. Nevertheless, heterokaryotypes are in excess, possibly caused by heterokaryotype advantage selection. We discuss our results in the light of general issues concerning the long-term maintenance of adaptive polymorphism.

2 | MATERIALS AND METHODS

2.1 | Samples

We analysed data from 1,360 *Timema cristinae* from 21 populations throughout the species range, which were sampled in spring 2013 and preserved in ethanol (Figure 2; Table S1). To study in detail the mechanisms maintaining colour and pattern morphs within populations and their genomic outcomes, we first focused analyses on a single site for which we had a large sample size ($n = 435$) and where *Adenostoma* and *Ceanothus* host plants co-occur. This population, named N1 (N34°31.034', W119°47.790'), comprises an area of about 50×70 m and has not previously been studied. Using sweep nets, we collected from N1 a total of 94 and 341 *T. cristinae* on 32 *Adenostoma* and 64 *Ceanothus* plant individuals, respectively. We then tested whether our findings can be replicated by reanalysing a second population with a large sample size (FHA), using data from 600 previously published samples (Comeault et al., 2015). *Adenostoma* dominates this site, and all *T. cristinae* were collected from this host. We detected major chromosomal variants associated with colour morphs in both populations. We thus investigated whether these were geographically widespread using previously published data from 19 additional populations sampled on either *Adenostoma* or *Ceanothus* throughout the species range (5–20 individuals per population, 325 in total; Figure 2; Table S1; Riesch et al., 2017).

2.2 | Phenotype characterization

Using digital photographs, we scored dorsal colour as "melanistic" or "green" and dorsal pattern in green individuals as "green-striped" or "green-unstriped". Because photographs were taken of most, but not all, collected *T. cristinae*, sample sizes were lower than for genetic data: 409 mostly sexually immature *T. cristinae* from N1, 588 adult samples from FHA (Comeault et al., 2015) and 305 adult samples from 18 additional populations (Riesch et al., 2017). For detailed analyses in N1 and FHA, we further classified phenotypes as "green-incomplete" if a dorsal stripe was present but faint or not developed along the full body length. Depending on the markedness of the stripe, these phenotypes were scored as either "green-striped" or "green-unstriped" otherwise.

For FHA, we also analysed a number of previously published continuous trait measurements on sexually mature individuals (Comeault et al., 2015; Riesch et al., 2017): per cent of the dorsal body area striped (% striped), body length (BL) and the following six continuous traits on colour channels: lateral green-blue, lateral red-green, lateral luminance (i.e., brightness), dorsal green-blue, dorsal red-green and dorsal luminance. We could not obtain these measurements for N1 because standardized photographs of adult individuals were not taken.

2.3 | Genotyping by sequencing

We obtained genomic DNA from all 435 specimens from N1 and prepared individually barcoded restriction-site-associated DNA

libraries using protocols as for the other previously published samples (Comeault et al., 2015; Riesch et al., 2017). Libraries were single-end-sequenced on three Illumina HiSeq2000 lanes at the National Center for Genome Research (Santa Fe, NM, USA). We filtered raw sequences and used BOWTIE2 (Langmead & Salzberg, 2012) to map reads to the *T. cristinae* reference genome (Riesch et al., 2017; Soria-Carrasco et al., 2014), which comprises 13 linkage groups (LGs), likely corresponding to the chromosomes of the species ($2n = 25/26, X0/XX$; Schwander & Crespi, 2009). We called variants using SAMTOOLS and BCFTOOLS (Li, 2011), and after discarding variants where <90% of samples were covered, we retained 304,168 biallelic SNPs with mean coverage depth per SNP per individual of $\sim 5\times$.

We reanalysed sequence data for 600 individuals from FHA (NCBI BioProject PRJNA284835; Comeault et al., 2015), after excluding two individuals with low sequence coverage. We called and filtered variants as above and retained 384,611 biallelic SNPs with mean coverage depth per SNP per individual of $\sim 7\times$.

We combined sequences from 325 *T. cristinae* that were sampled from 19 additional populations distributed across the species range (NCBI BioProject PRJNA356885; Riesch et al., 2017) with 20 individuals from each N1 and FHA. Samples from N1 and FHA were chosen such that all main karyotypes (below) were included, and served as references to determine whether the same karyotypes were present across the species range. We used settings for sequence filtering, mapping and variant calling as above and retained 626,854 biallelic SNPs with mean coverage depth per SNP per individual of $\sim 5\times$. Further details are provided in Appendix S1.

2.4 | Identification of genomic clusters

2.4.1 | Principal component analysis

We conducted principal component analysis (PCA) separately for populations N1, FHA and the combined data set of 21 populations. Because missing genotype information can affect principal components, we first recalled SNPs for PCA analyses, requiring at least 99% of individuals to be covered, retaining 62,542, 168,020 and 99,008 SNPs, respectively. To account for genotype uncertainty, we used a Bayesian model and Markov chain Monte Carlo to obtain joint posterior probabilities for genotypes and allele frequencies given the genotype likelihoods estimated by BCFTOOLS, along with Hardy–Weinberg priors, as in past work (Gompert et al., 2012, 2014; Nosil et al., 2012). We further excluded variants with a minor allele frequency (MAF) <1%, and randomly selected SNPs to achieve at least 100 bp distance among variants, retaining 11,751, 30,297 and 8,758 SNPs for N1, FHA and the combined data set, respectively.

We collapsed posterior genotype probabilities into a single value per individual and locus (i.e., posterior mean of alternative allele dosage, ranging from zero to two), centred values for each SNP by subtracting the mean over all individuals and conducted a PCA on the genotype covariance matrix on the centred but unscaled values using the *prcomp* function in R (R Core Team, 2016). Visual

inspection of PCA scatter plots revealed three striking genotypic clusters on the first two PC axes (Figs S1a and S2a). To investigate this clustering in more detail, we sequentially removed 48 and 37 genomewide PCA outliers as in Price et al. (2006) for populations N1 and FHA, respectively. Briefly, PCAs were visually inspected after each iteration of outlier removal and remaining samples were subjected to a new iteration until individuals peripheral of the main clusters were eliminated (Figs S1 and S2; more details on the process of outlier removal and discussion of outliers in Appendix S1). PCA applied separately to each LG revealed that genomic clustering could be attributed to variation on LG8 only and that the three main clusters further split on LG8 into a total of six clusters (Figure 1b; Figs S1–S5). We defined these PCA clusters for N1 and FHA by first grouping individuals by *k*-means clustering on the first 10 PC axes computed from SNPs on LG8 only (*kmeans* function in R, with 10 initial centres). We then obtained assignment probabilities for individuals per cluster by applying linear discriminant analysis of the first 10 PC axes as explanatory variables and cluster assignment as grouping factor (*lda* function in R, MASS library; leave-one-out cross-validation) and retained samples with at least 80% assignment probability to any cluster for further analyses (Figure 1c; Tables S2 and S3; Fig. S4b). We then tested for an association between PCA clusters and phenotypic morphs using chi-squared tests with the *chisq.test* function in R, and significance values were computed by Monte Carlo simulation with 100,000 replicates.

2.4.2 | Model-based cluster assignment

Given the distinctive arrangement of PCA clusters on only one LG, their association with colour and pattern morphs and the known dominance relationships for colour and pattern loci (Comeault et al., 2015; Sandoval, 1994a,b), we suspected that clusters were caused by divergent chromosomal variants existing as homo- and heterokaryotypes (i.e., heterokaryotypes are located in-between the homokaryotypes in the PCA plot). We thus predicted that by assigning diploid genomic ancestry to each locus and individual, hetero- or homozygous ancestries would prevail for genomic regions causing these clusters (e.g., three main PCA clusters could then be described by diploid combinations of two ancestry clusters “melanistic” and “green”, Figure 1d, and six PCA clusters by diploid combinations of three ancestry clusters “melanistic”, “green-striped” and “green-unstriped”, Figure 1c).

We used the software STRUCTURE to obtain locus-specific estimates of ancestry for SNPs on LG8 (the site-by-site output from the linkage model; Falush, Stephens, & Pritchard, 2003; Pritchard, Stephens, & Donnelly, 2000). To test whether individuals from different PCA clusters represent homozygous and heterozygous combinations of two main ancestry clusters of which one is further subdivided, we set the number of ancestry clusters to $k = 2$ or $k = 3$. To obtain karyotype assignments for tests of Hardy–Weinberg equilibrium (HWE) and phenotypic differences among karyotypes (below), we also ran STRUCTURE using all individuals from populations N1 or FHA (i.e., including PCA outliers; 435 or 600 samples) using SNPs on

three adjacent scaffolds on LG8 that showed a particularly strong signal of genetic clustering ("scaffolds 931, 318 and 1440" hereafter). We set $k = 2$ as we were interested in karyotype estimates for the main axis of variation (i.e., "melanistic" vs. "green" variants). We repeated this analysis for the combined data set of 21 populations to test whether variants are geographically spread and in HWE. Further details of preparation of STRUCTURE input files and settings are provided in Appendix S1.

2.5 | Multilocus genomewide association mapping

To map colour and pattern traits, we used population FHA where we had a better record of phenotypic traits and a larger number of samples than for N1. Although these traits have previously been mapped in FHA (Comeault et al., 2015, 2016; Riesch et al., 2017), we remapped them here with the sets of SNPs and individuals used in our other analyses to make results compliant (i.e., using the same version of the *T. cristinae* reference genome and excluding PCA outliers). We excluded individuals with ambiguous phenotype data (i.e., classified differently by two researchers), and scored both colour and pattern as binary traits. We restricted mapping to SNPs assigned to LGs and with $MAF \geq 1\%$, retaining 180,512 SNPs and 552 samples for colour, and 180,506 SNPs and 498 samples for pattern mapping (only green individuals were used for the latter). We mapped traits using Bayesian sparse linear mixed models with the probit model implemented in the software GEMMA (Zhou, Carbonetto, & Stephens, 2013), as in previous work (details in Appendix S1; Comeault et al., 2015, 2016; Riesch et al., 2017).

2.6 | Population genomic statistics

We found that the six PCA clusters were indeed associated with homo- and heterokaryotypic combinations of three chromosomal variants (see Section "3"). To obtain information regarding the evolutionary processes affecting these variants and the time scales involved, we computed and compared different population genomic statistics. This was performed across the genome within or between PCA clusters, depending on the prediction being tested. Specifically, we estimated relative and absolute between-cluster divergence (F_{ST} and D_{xy} , respectively), within-cluster nucleotide diversity (π), and a measure of between-cluster, intralocus LD (Z_g ; Storz & Kelly, 2008). In addition, we surveyed chromosomal variants for signals of recent positive selection ("selective sweeps") by estimating extended haplotype homozygosity within and between clusters (iES and Rsb; Tang, Thornton, & Stoneking, 2007). All statistics were computed in nonoverlapping 20-kb windows. We provide below an overview of the logic behind our analyses with details of how the statistics were calculated provided in Appendix S1.

Balancing selection might target a single locus, multiple loci, or structural genomic changes such as chromosomal inversions. The genomic processes of balancing selection and their expected outcomes arising at or linked to inversion breakpoints are highly similar to those expected for a single or multiple linked selected loci. Loci

subject to long-term varying selection are expected to show elevated nucleotide diversity between alleles sampled from different subpopulations (or here, different chromosomal variants) relative to diversity within them (Charlesworth, Nordborg, & Charlesworth, 1997; Hudson & Kaplan, 1988; Kelly & Wade, 2000; Nordborg & Innan, 2003; Storz & Kelly, 2008). In contrast, new alleles that were rapidly driven to high or intermediate frequencies by selection will show reduced diversity compared to ancestral alleles or neutral loci (Sabeti et al., 2002; Voight, Kudaravalli, Wen, & Pritchard, 2006). Similarly, for a sufficiently old inversion polymorphism maintained by balancing selection, alleles of sites linked to inversion breakpoints are expected to show longer coalescent times (i.e., elevated π ; Wakeley, 2008) when sampled from heterokaryotypes compared to either genomewide expectations or alleles sampled from any homokaryotype (Guerrero, Rousset, & Kirkpatrick, 2012; Navarro, Bardadilla, & Ruiz, 2000). In contrast, the evolution of a new inversion will eliminate diversity within inversion homokaryotypes, which will only slowly recover through genetic exchange with the standard type. As recombination is more likely in the centre of the inversion, reduced diversity will remain near the breakpoints until new mutations accumulate (Guerrero et al., 2012; Navarro, Betran, Barbadilla, & Ruiz, 1997; Navarro et al., 2000).

In addition, increased levels of LD are expected to build up at and closely linked to selected loci or inversion breakpoints that are maintained by balancing selection (Charlesworth et al., 1997; Kelly, 1997; Peischl, Koch, Guerrero, & Kirkpatrick, 2013; Storz & Kelly, 2008; Wallace, Detweiler, & Schaeffer, 2013). LD can also extend over larger genomic regions following a selective sweep, but is expected to decay over time with increasing physical distance from single selected loci (Sabeti et al., 2002; Slatkin, 2008), and at a slower rate for multiple linked selected loci or inversions where recombination is suppressed (Navarro & Barton, 2002; Peischl et al., 2013; Wallace et al., 2013).

We were interested in detecting genetic regions subject to balancing selection (including putative breakpoints of inversions that might be associated with divergent chromosomal variants), and in investigating whether chromosomal variants show indications for recent or ancient evolution. We thus examined the genome for heterogeneity in Z_g , D_{xy} , and Rsb between homokaryotypic clusters, and compared π in homokaryotypic clusters to π in their corresponding heterokaryotypic cluster along the genome. To facilitate comparison among the different statistics we used, we defined "high-differentiation scaffolds" for each pair of homokaryotypic cluster as scaffolds with mean F_{ST} above or equal the 97.5% quantile of all scaffolds from the 13 LGs. Scaffolds 931, 318 and 1440 (above) are a subset of these high-differentiation scaffolds (Figure 3 shows their positions on LG8).

To further investigate the mechanisms and the history of balancing selection, we computed additional statistics for whole populations, irrespective of genetic clusters. We contrasted statistics that are informative regarding balancing selection in the sampled generation (i.e., HWE) and those indicative of such selection in the recent or distant past (i.e., LD and Tajima's D ; Garrigan & Hedrick, 2003;

Hedrick, 2006). Specifically, we computed Burrow's composite measure of within-population LD (Δ ; Weir, 1979) between pairs of SNPs, and Tajima's D (Tajima, 1989) in nonoverlapping 20-kb windows. Increased LD can indicate that multilocus balancing selection acted consistently during the recent history of populations (Garrigan & Hedrick, 2003; Hedrick, 2006; Navarro & Barton, 2002). Positive values of Tajima's D can arise if mutations accumulated independently among polymorphic variants, which is expected if balancing selection acted over extended periods of time in the distant past (Garrigan & Hedrick, 2003; Hedrick, 2006; Simonsen, Churchill, & Aquadro, 1995). As positive Tajima's D can potentially also result from directional positive selection that differs from the standard full-sweep model (Przeworski, Coop, & Wall, 2005), we used linear regression to test whether increased Tajima's D is associated with decreased π or increased iES in any of the homokaryotypic clusters, indicating that recent positive selection on one chromosomal variant might have caused an excess of intermediate-frequency alleles in the whole population and increased Tajima's D . Because Tajima's D or LD can equally be affected by recent population dynamics, we compared these statistics computed for different scaffolds on LG8 to genomewide expectations.

2.7 | Divergence dating

We estimated divergence time between chromosomal variants by two different methods. First, we used the program BEAST 2 (Bouckaert et al., 2014), including previously published genetic data and divergence times of four *Timema* species related to *T. cristinae* (NCBI BioProject PRJNA356405; Riesch et al., 2017). We based estimations on scaffolds 931, 318 and 1440 or on high-differentiation scaffolds common to all three pairwise combinations of homokaryotypic clusters, without assuming a chromosomal inversion. Second, we used Approximate Bayesian Computation (ABC), based on scaffolds 931, 318 and 1440, assuming the presence of a chromosomal inversion (details in Appendix S1).

2.8 | Tests for HWE and heterokaryotype excess

To further test for balancing selection in the sampled generation, we tested for heterokaryotype excess relative to HWE. We classified individuals as homo- or heterokaryotypes of the two main chromosomal variants "melanistic" and "green", given their diploid genomic ancestry on scaffolds 931, 318 and 1440 assigned by STRUCTURE and $k = 2$ (above). To define karyotypes, we used thresholds for STRUCTURE admixture proportions (q) that best delimited clusters for each data set ($0.3 < q < 0.7$, $0.32 < q < 0.68$ and $0.38 < q < 0.62$ for N1, FHA and the combined data set, respectively; Fig. S6). We used the obtained karyotype counts to apply an exact test for HWE (Wiggin-ton, Cutler, & Abecasis, 2005), using R code from http://csg.sph.umich.edu/abecasis/Exact/r_instruct.html. We further measured the direction of deviation from HWE using the fixation index $F = (H_E - H_O)/H_E$, where H_E and H_O are the expected and observed heterokaryotype frequencies.

2.9 | Mating preference models

We used a modelling approach to test to what extent heterokaryotype advantage selection or mating preferences likely contribute to the observed frequencies of main karyotypes. We considered two models, one of negative assortative mating and one of universal mating advantage of the melanistic morph (Comeault et al., 2015). We assumed that m and G variants correspond to a single locus with two alleles associated with melanistic and green morphs and controlling mating preferences, and with selection acting on one or both homokaryotypes. We first computed equilibrium genotype proportions for various strengths of mating preference and selection and then obtained the probability of sampling the genotype counts observed in N1 or FHA from these proportions (details in Appendix S1).

2.10 | Test for phenotypic differences among karyotypes

We used linear modelling to investigate whether phenotypic traits (i.e., BL and six continuous colour traits measured for individuals from FHA) are associated with karyotype. Individuals were classified as homo- and heterokaryotypes for the "green" and "melanistic" variants as before. We determined whether adding either karyotype or binary colour state as explanatory variable improved models by analysis of deviance and by difference in Akaike's information criterion (ΔAIC ; including sex and % striped as covariates; details in Appendix S1).

3 | RESULTS

We first report results of genomic analyses in population N1, and then complement them with analyses involving phenotype measurements, or karyotype assignments across the species range. Unless stated otherwise, results reported in the main article were obtained from N1 as only this population had all three chromosomal variants present as homokaryotypes in adequate quantities. Comparable results from FHA are provided in Appendix S1.

3.1 | Phenotypic morphs are associated with highly divergent genetic clusters

The first axis of variation in the genomewide PCA analysis showed three striking genotypic clusters that were almost perfectly associated with colour morph (i.e., two green clusters and one melanistic cluster; x-axis in Fig. S1f). This clustering by colour was explained by LG8 only (Fig. S3), and PCA restricted to variants on LG8 revealed additional clustering on the second axis of variation that was associated with pattern morphs (Figure 1b).

We assigned individuals to the six conspicuous clusters on the first two PC axes using a model-free clustering algorithm. These clusters showed a pronounced nonrandom association with phenotypic morphs ($\chi^2_{(15, N=357)} = 647$, p -value = 0.00001, Cramér's

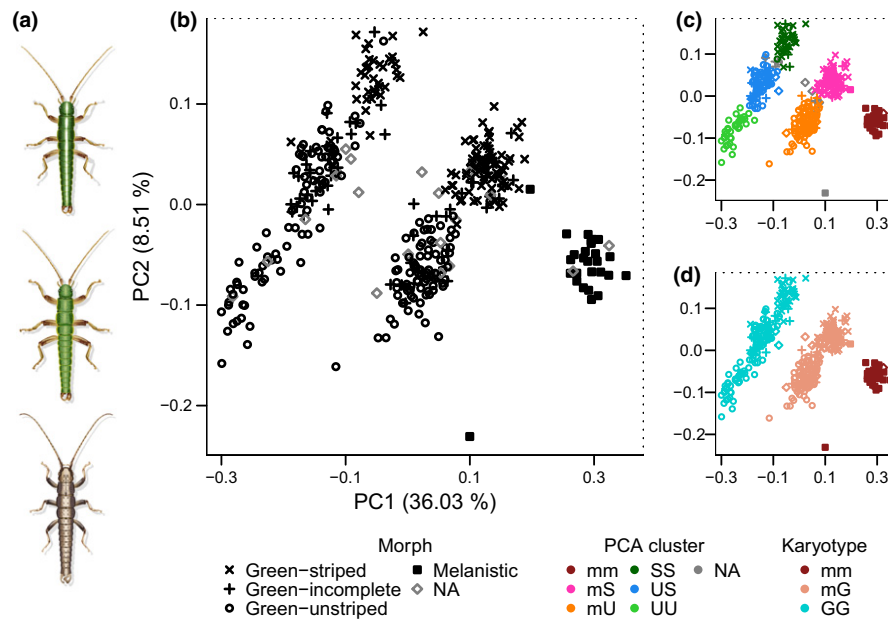
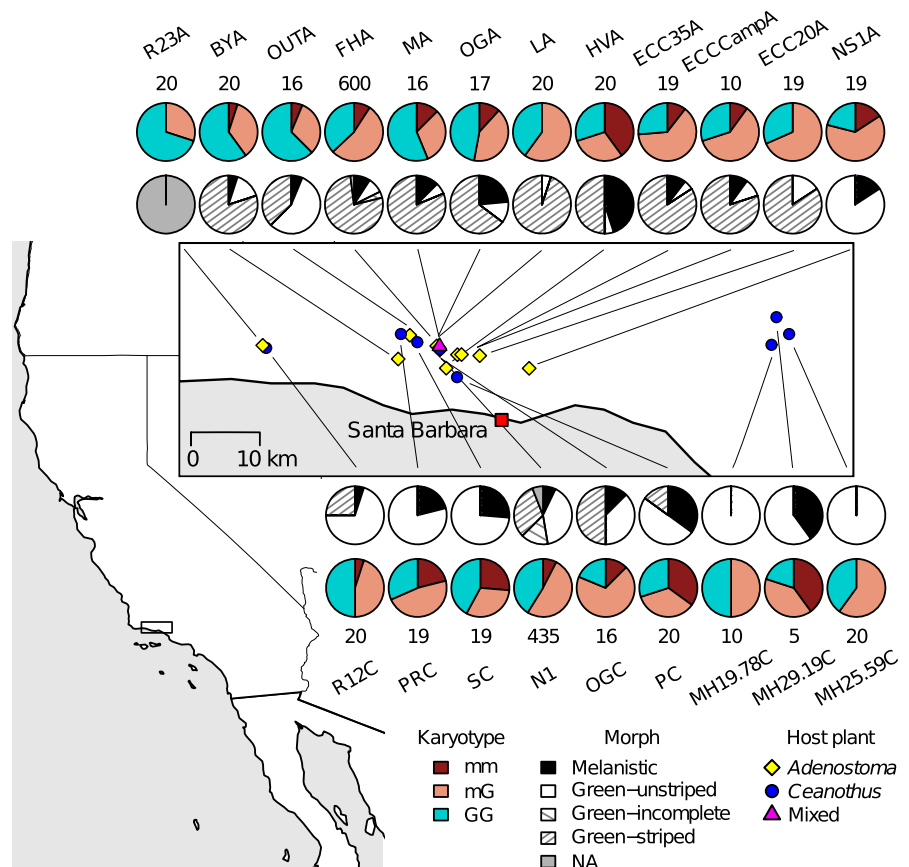


FIGURE 1 Genetic structure on LG8 associated with phenotypic morphs in population N1. (a) Three *Timema cristinae* morphs cryptic on leaves or stems of their host plants: green-striped, green-unstriped and melanistic. (b) Principal component axis one (PC1) shows clustering by colour morph, with two distinct clusters for green morphs (crosses, pluses and circles) and one cluster for the melanistic morph (filled squares). Principal component axis two (PC2) shows a gradient by pattern morph, from green-unstriped (circles) to green-striped morphs (crosses). (c) K-means clustering and linear discriminant analysis were used to define six PCA clusters, corresponding to diploid combinations of three chromosomal variants “melanistic” (m), “green-striped” (S), and “green-unstriped” (U). (d) STRUCTURE with $k = 2$ identified two main chromosomal variants “melanistic” (m) and “green” (G), resulting in three main karyotypes by their diploid combinations. PCA outlier individuals were excluded in (b) and (c) and are not shown in (d)

FIGURE 2 Sampling localities, karyotype and morph frequencies for 21 *Timema cristinae* populations. Samples were obtained from 12 populations on *Adenostoma* host plants (yellow diamonds), eight populations on *Ceanothus* host plants (blue circles) and one mixed population (N1, magenta triangle) across the species distribution around Santa Barbara, California. Pie charts in the top and bottom rows show karyotype frequencies estimated by genetic clustering using STRUCTURE with $k = 2$ (i.e., corresponding to m and G chromosomal variants). Pie charts in the second and third rows give morph frequencies for *Adenostoma* hosts (top) and *Ceanothus* or mixed hosts (bottom). Population names and sample sizes are indicated



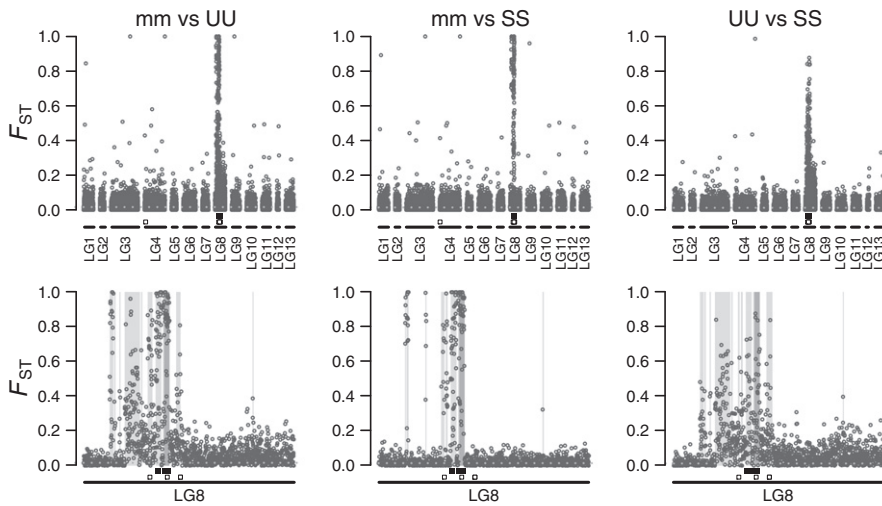


FIGURE 3 Genomewide differentiation between pairs of homokaryotypic clusters in population N1. Top row, F_{ST} for nonoverlapping 20-kb windows and all LGs; bottom row, LG8 only. Grey dotted lines show genomewide 50% quantiles, and squares on the x-axis indicate positions of candidate SNPs for colour (closed symbols) and pattern (open symbols). High-differentiation scaffolds are highlighted in grey, with a darker shade indicating a subset of three scaffolds that were treated separately in some analyses (scaffolds 931, 318 and 1440). The distinctive blocks of high F_{ST} on LG8 span approximately 13 Mb

$V = 0.78$; Figure 1c; Table S2). We suspected that the observed structure was caused by three divergent chromosomal variants associated with colour and pattern and existing in homo- and heterokaryotypic combinations. We refer to these variants and resulting clusters as follows: (i) three chromosomal variants m, U and S (i.e., “melanistic”, “green-unstriped” and “green-striped”); (ii) six PCA clusters mm, UU, SS, mU, mS and US, resulting from all six possible homo- or heterokaryotypic combinations of these chromosomal variants (Figure 1c); and (iii) three main karyotypes mm, mG and GG, resulting from STRUCTURE analysis with $k = 2$ (i.e., “green-unstriped” and “green-striped” variants pooled as “green”; Figure 1d). We provide further support for our notations below.

3.2 | Genetic differentiation between clusters

To more finely determine the genomic regions generating genetic clustering, we estimated genomewide F_{ST} between pairwise combinations of homokaryotypic clusters (mm, UU and SS). This revealed that F_{ST} between clusters ranged among 20-kb windows from values near zero to one, but that strong differentiation was almost entirely restricted to one region of ~13 megabases of sequence covering ~29% of LG8 (Figure 3). Absolute divergence measured as D_{xy} (Figure 4) and joint allele frequency spectra (Fig. S7) further confirmed strong differentiation and putatively evolutionary independence between m and U or m and S variants. The observed block-like pattern of high F_{ST} is reminiscent of the genomic outcome of a chromosomal inversion that suppresses effective recombination and genetic exchange among chromosomal variants (e.g., Cheng et al., 2012; Corbett-Detig & Hartl, 2012; Lamichhane et al., 2016; Tuttle et al., 2016), although future work is needed to test this hypothesis directly.

3.3 | Genetic clusters represent distinct chromosomal variants

STRUCTURE analyses with $k = 2$ confirmed that the three main clusters on PC axis 1 represent homo- and heterozygous combinations of two ancestry clusters (i.e., m and G; Figure 1d; Figs S8 and S9). By

defining $k = 3$, we could also support that genomic clusters on PC axis 2 represent homo- and heterozygous combinations of a further subdivision of G identified with $k = 2$ (Figs S10 and S11). This is consistent with our expectation of three chromosomal variants m, U and S. Their associations with phenotype are in agreement with previous results on dominance and linkage within and among colour and pattern loci mapped to LG8 (Comeault et al., 2015). We further confirmed that our second population, FHA, showed patterns of genomic clustering and differentiation that were very similar to N1, although the UU karyotype was not identified and likely not strongly represented (Table S3; Figs S2, S4, S5 and S12), which prevented comparisons among all three homokaryotypic clusters.

3.4 | Multilocus genomewide association mapping

Multilocus genomewide association mapping in population FHA confirmed that candidate SNPs for colour and pattern are located within the boundaries defining divergent chromosomal variants on high-differentiation scaffolds on LG8 (except one pattern candidate SNP on LG4; Figure 3; Tables S4–S7). However, the specific positions of candidate SNPs should be interpreted very cautiously given the particularly high levels of LD in this region (below; Figures 4 and 5b; Fig. S13b). For this reason, we did not pursue further functional annotation of candidate SNPs.

3.5 | Chromosomal variants are ancient and present throughout the species range

The high level of divergence among chromosomal variants suggests that they have coexisted for a sufficient amount of time to build up genetic differentiation. To investigate the evolutionary history of the three chromosomal variants in more detail, we determined several statistics for homokaryotypic (mm, UU and SS) and heterokaryotypic clusters (mU, mS and US) that are informative regarding divergence time and the processes of selection. We restricted our analyses to LG8 given that genetic clustering and association with phenotypic morphs was largely confined to this part of the genome.

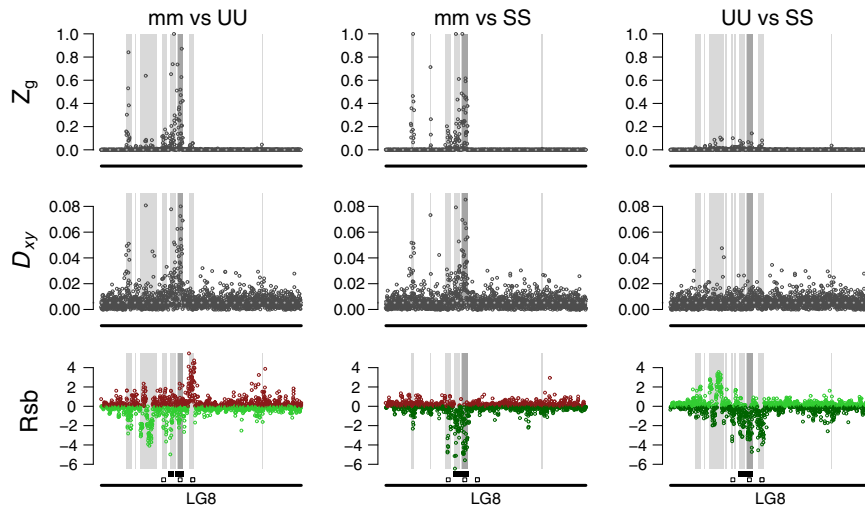


FIGURE 4 Population genomic parameters along LG8 for pairs of homokaryotypic clusters in population N1. Regions of high intralocus LD (Z_g) and increased absolute genetic divergence (D_{xy}) between clusters coincide with high-differentiation scaffolds (Figure 3; highlighted in grey). Extreme values in R_{sb} indicate that regions of extended haplotype homozygosity differ among clusters. All statistics were calculated in nonoverlapping 20-kb windows. Grey dotted lines show genomewide 50% quantiles for Z_g and D_{xy} , and squares on the x-axis indicate positions of candidate SNPs for colour (closed symbols) and pattern (open symbols)

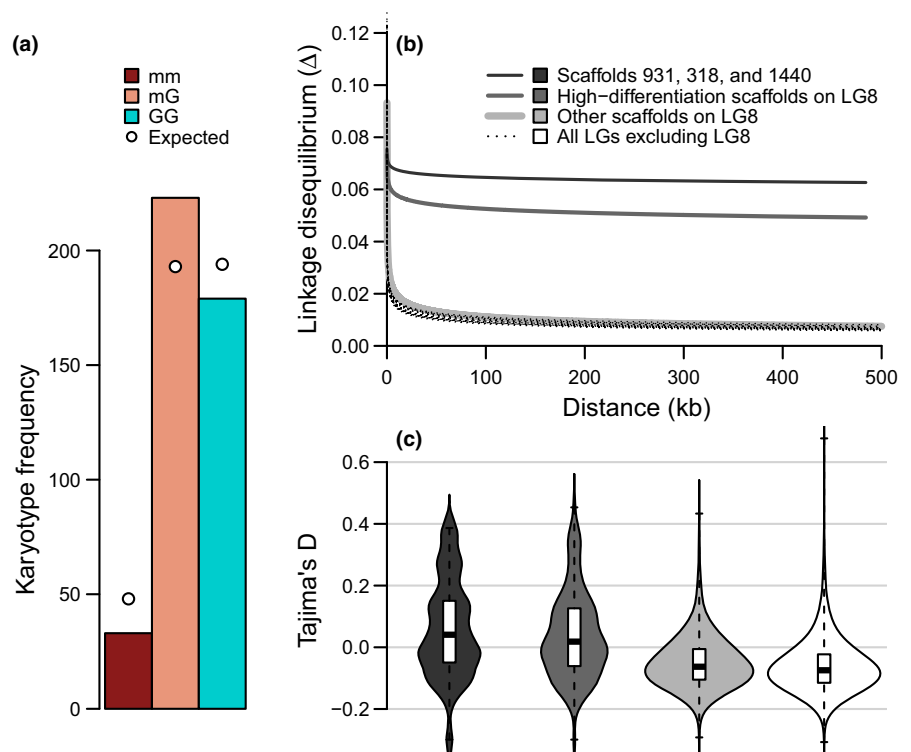


FIGURE 5 History of balancing selection in population N1. (a) Karyotype frequencies. Bars show observed karyotype counts, and circles indicate expected counts for a population in HWE. (b) Decay of LD with physical distance between pairs of SNPs summarized for different sets of scaffolds on LG8 and for all other LGs. The y-axis shows Burrow's composite measure of Hardy–Weinberg and LD (Δ). Lines were fitted by nonlinear regression. (c) Tajima's D statistic for nonoverlapping 20-kb windows for different sets of scaffolds on LG8 and for all other LGs combined. White boxes range from the first to third quartile, black horizontal bars give the median, whiskers extend to the data extremes and shapes are Gaussian kernel densities

We found that the genomic region of high F_{ST} between chromosomal variants also showed considerably elevated D_{xy} and Z_g between the melanistic variant (m) and either green variant (U or S), compared to genomewide expectations (Figure 4). This suggests the region was subject to varying selection between variants (Storz & Kelly, 2008). Similarly, π within mU and mS heterokaryotypes was elevated, while π within mm and UU homokaryotypes approached background levels of diversity, resembling the expected outcomes

for an old inversion polymorphism maintained by balancing selection (Figure 6; Navarro et al., 2000; Guerrero et al., 2012). Nevertheless, mm and UU homokaryotypes also showed deviations in π and R_{sb} along LG8, consistent with the effects of more recent differential selection (Figures 4 and 6; Fig. S14).

In contrast with the pronounced differentiation between the melanistic and either green variant, when we compared the two green variants, U and S, we found that D_{xy} and Z_g were only slightly

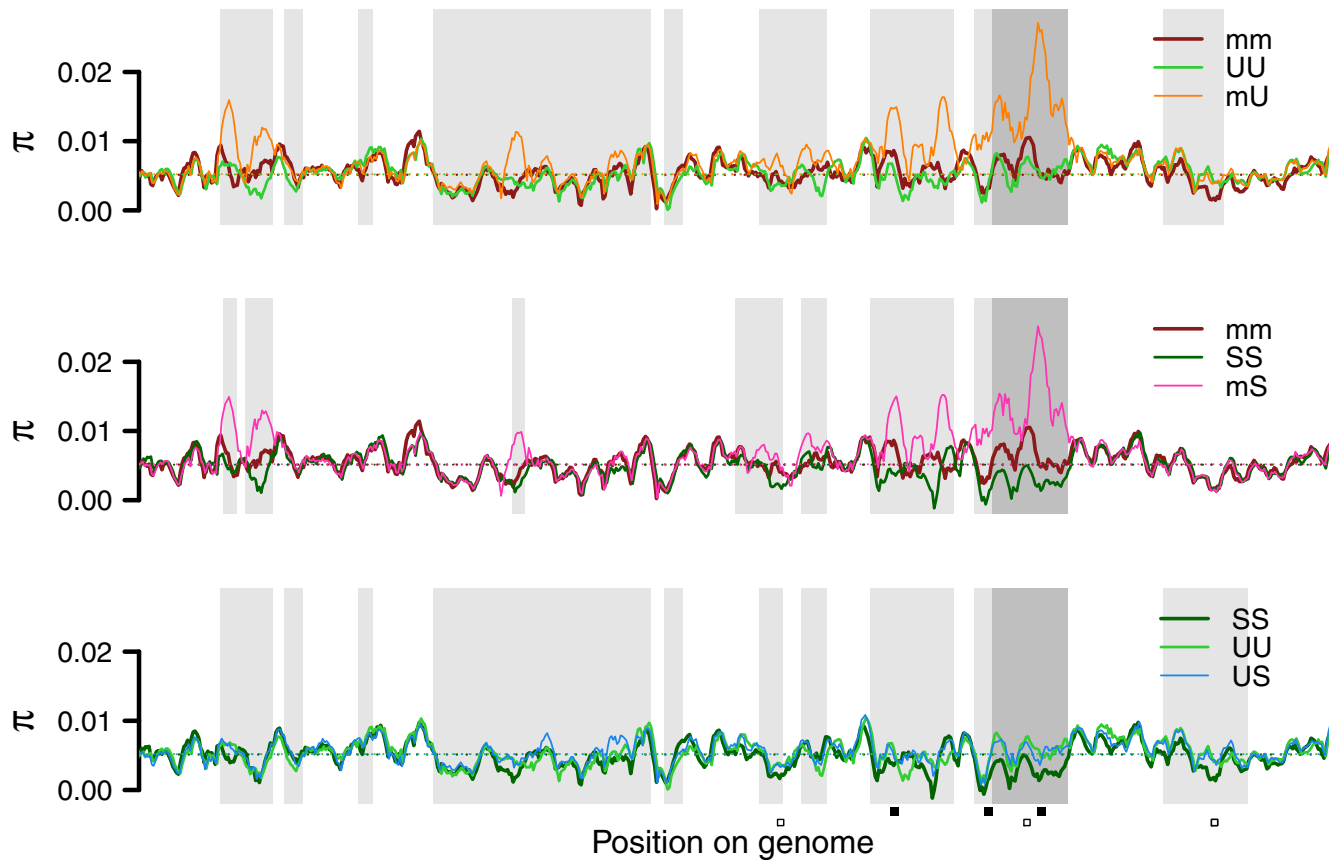


FIGURE 6 Nucleotide diversity (π) along LG8 for all clusters in population N1. Approximately one-third of LG8 is shown. Each panel shows π for two homokaryotypic clusters and their corresponding heterokaryotypic cluster. Orange and pink lines in the top two panels show regions of increased diversity in heterokaryotypes between melanistic and green variants (mU or mS) relative to diversity within homokaryotypes (mm, UU or SS), coinciding with high-differentiation scaffolds (Figure 3; highlighted in grey). Increased diversity is not observed for heterokaryotypes between green-unstriped and green-striped variants (US; bottom panel, blue line). Lines depict smoothed estimates from nonoverlapping 20-kb windows. Dotted lines show genomewide 50% quantiles, and squares on the x-axis indicate positions of candidate SNPs for colour (closed symbols) and pattern (open symbols)

elevated compared to genomewide expectations (Figure 4). Increased haplotype homozygosity and significantly reduced levels of π within SS homozygotes further suggest that the green-striped variant experienced a considerable recent selective sweep (Figures 4 and 6; Fig. S14). Our results thus support a recent evolution of the green-striped variant, consistent with a young polymorphism or new chromosomal inversion (DeGiorgio, Lohmueller, & Nielsen, 2014; Guerrero et al., 2012; Navarro et al., 2000).

We further evaluated the age of the chromosomal variants using BEAST 2 and ABC. BEAST 2 analyses estimated m and U variants to have split 13.5 or 8.0 million years (Ma) ago, based on scaffolds 931, 318 and 1440 or on high-differentiation scaffolds, respectively (95% highest posterior density intervals: 2.3–20.0 or 2.3–15.2 Ma; Table S10; Fig. S15; 1 year corresponds to one generation in *T. cristinae*). By contrast, we estimated U and S variants to have split more recently, 2.7 or 1.8 Ma ago, based on the two sets of scaffolds, respectively (95% highest posterior density intervals: 0.6–5.7 or 0.7–3.3 Ma). When using ABC, meaningful time estimates were not possible due to a wide spread of the posterior distribution (median: 0.87 million generations, 2.5% and 97.5%

quantiles: 0.018 and 39.3 million generations; Figs S16 and S17). This might reflect uncertainty in parameter estimates affecting divergence time, or an old polymorphism has reached equilibrium and thus provides little information on divergence time under a simple mutation model.

A sufficiently old and balanced polymorphism might be spread through large parts of the species range. To test this expectation, we reanalysed samples from 19 localities across the species distribution. Isolation-by-distance contributes to pronounced genetic divergence among *T. cristinae* populations (Nosil et al., 2012; Riesch et al., 2017), rendering analyses of differentiation between the only slightly differentiated U and S chromosomal variants difficult (Fig. S18). We thus considered here only the two main chromosomal variants (m and G) identified by STRUCTURE with $k = 2$. We found that m variants were indeed present in all populations at considerable and similar frequencies (mean 0.37, SD 0.12; Figure 2; Table S1). This finding is consistent with geographically widespread balancing selection within populations, as opposed to gene flow–selection balance among divergent populations. It also suggests that balancing selection may have acted for an extended period of time, particularly given the low

dispersal distance of *T. cristinae* (i.e., metres to dozens of metres per generation; Sandoval, 2000) and the patchiness of the habitat.

3.6 | Further consideration of the mechanisms of balancing selection

We investigated the mechanisms and the history of balancing selection using additional population genetic parameters that are informative regarding this type of selection at different time scales. To determine the potential mechanisms of balancing selection in the current generation, we tested for deviations from HWE for the two main chromosomal variants *m* and *G*. In N1, we found that heterokaryotypes were in considerable excess relative to their expected frequency (15.5% more heterokaryotypes than expected; fixation index $F = -0.16$, p -value = 0.00119; Figure 5a; Table S1). FHA showed a similar heterokaryotype excess (16.7% more than expected; $F = -0.17$, p -value = 0.00004; Table S1; Fig. S13a). Estimates from additional populations across the species range suggest that heterokaryotype excess could be widespread, although not necessarily ubiquitous (10 of 19 populations showed $F < 0$; Table S1). Although low sample sizes preclude a definitive test for HWE in all 19 populations, heterokaryotype excess is clearly evident in both populations for which we had large sample sizes.

To examine whether balancing selection acted in the past, we calculated within-population LD (Δ) and Tajima's D . We found increased Δ ranging over several hundreds of kb distance between SNPs for high-differentiation scaffolds, relative to other scaffolds (Figure 5b; Fig. S13b). This is consistent with high levels of between-cluster, intralocus LD (Z_g) determined above, which measures a different aspect of LD. Further, Tajima's D was elevated for high-differentiation scaffolds compared to other scaffolds on LG8 or genomewide expectation (Figure 5c; Fig. S13c; p -value = 5.076×10^{-15} or $< 2.2 \times 10^{-16}$, Mann–Whitney U tests). We found no evidence that increased Tajima's D in high-differentiation scaffolds was caused by recent positive selection on any chromosomal variant (Figs S19 and S20). Our results thus indicate that balancing selection maintained the polymorphism on LG8 during the past. As an extended genomic region shows these signals of balancing selection, our results further indicate that selection targets many linked loci or a region of strongly reduced recombination (Kelly & Wade, 2000; Navarro & Barton, 2002; Nordborg & Innan, 2003).

3.7 | Testing for potential causes of heterokaryotype excess

The observed heterokaryotype excess could arise through two main and potentially overlapping mechanisms: negative assortative mating or heterozygote advantage selection. Our data suggest that negative assortative mating between melanistic and green morphs is unlikely to have caused the heterokaryotypes excess. Specifically, the equilibrium frequency of the recessive colour allele is expected to be ~ 0.71 for various strengths of negative assortative mating (Hedrick et al., 2016). However, we observe much lower frequencies of 0.33 and 0.36 for *m* variants in populations N1 and FHA, respectively

(assuming here that *m* and *G* chromosomal variants are perfectly associated with colour morphs, which is largely consistent with our results). Moreover, mating trials do not support negative assortative mating in *T. cristinae*, where, if anything, melanistic morphs have a universal mating advantage (Comeault et al., 2015). We further tested which strengths of heterokaryotype advantage selection and mating preferences could explain the observed karyotype frequencies in N1 and FHA. A model for negative assortative mating and one for universal mating advantage of the melanistic morph both indicate that heterokaryotype advantage selection likely contributes generating the observed frequencies, although we cannot fully exclude alternative scenarios (Figs S21 and S22).

Given some evidence for heterokaryotype advantage, we tested whether heterokaryotypes differ from homokaryotypes in traits known to affect survival in *T. cristinae* (Nosil & Crespi, 2006). We found that karyotypic state had a minor but significant effect on BL and all continuous colour traits tested (after Benjamini and Hochberg adjustment; Table S8; Fig. S23). The effect of karyotype remained significant with models only addressing variation between green morphs with *mG* versus *GG* karyotypes (Table S9). Future work is required to determine whether phenotypic differences among karyotypes affect fitness and contribute to heterokaryotype excess.

4 | DISCUSSION

Timema cristinae exhibits three colour and pattern morphs that are cryptic on different plant parts and on different plant species. The frequent co-occurrence of melanistic and green colour morphs on the same host plants allowed us to address the putative duration and evolutionary mechanisms maintaining the cryptic polymorphism within populations of this species. Despite genetic drift and changing selection pressures being expected to eventually lead to the loss of existing variants (Charlesworth & Charlesworth, 2010), our results support that colour morphs have been maintained over extended periods of time by balancing selection. We have revealed that the colour polymorphism is associated with highly divergent chromosomal variants involving several megabases of sequence. Interestingly, our results suggest that heterokaryotype advantage might contribute to maintaining this chromosomal polymorphism. This is surprising because incomplete dominance or recombination in heterokaryotypes might result in maladaptive intermediate phenotypes that do not match either stems or leaves of either host plant. We here discuss four aspects of our results: (i) the genetic architecture of cryptic; (ii) the maintenance of polymorphisms through time; (iii) the mechanisms of that maintenance; and (iv) the implications for adaptation and speciation.

4.1 | Genetic architecture of cryptic

The genetic architecture of cryptic colour and pattern polymorphism in *T. cristinae* agrees with two main observations of the

genetic basis of discrete colour polymorphisms in a variety of organisms (Laurens, Whibley, & Joron, 2017). First, colour and pattern exhibit dominance hierarchies (also see Comeault et al., 2015; Sandoval, 1994a,b), in line with findings in other organisms (Clarke & Sheppard, 1972; Johannesson & Butlin, 2017; Joron et al., 2011; Le Poul et al., 2014). Second, in several species, colour polymorphisms were mapped to regions of reduced recombination such as chromosomal inversions or supergenes (Joron et al., 2011; Kuepper et al., 2016; Kunte et al., 2014; Lamichhaney et al., 2016; Richards et al., 2013; Tuttle et al., 2016; Wellenreuther, Svensson, & Hansson, 2014). Our results revealed that in *T. cristinae*, colour and pattern traits are associated with an extended genomic region, consistent with highly reduced recombination. Patterns of Z_g and π further indicate that recombination is reduced between different karyotypes relative to within them, consistent with the presence of a chromosomal inversion, as are the particular patterns of F_{ST} and π along the genome (Figures 3, 4 and 6). Nevertheless, if selection targets many linked loci so that recombination among them is effectively reduced through low fitness of recombinants, similar outcomes are expected without an inversion (Kelly & Wade, 2000; Navarro & Barton, 2002; Nordborg & Innan, 2003). Future work is required that explicitly test for the presence of a chromosomal inversion, and whether different variants are the result of several linked inversions (e.g., Joron et al., 2011) or evolved by rare recombination events in heterokaryotypes (e.g., Imsland et al., 2012). Further, studies on the cytogenetics of the genus that extend previous work (Schwander & Crespi, 2009) and that determine fitness effects of crossover events in inversion heterozygotes are needed.

The agreement in the genetic architecture of crypsis in *T. cristinae* with that of colour-polymorphic traits in various other species suggests that architectures that prevent formation of maladapted phenotypic intermediates without reducing gene flow genomewide might be a common evolutionary outcome. The presumably opposing selective advantage of melanistic and green morphs on different plant parts and the seeming absence of positive assortative mating between them is theoretically expected to select for chromosomal rearrangements that reduce recombination between locally adapted alleles at multiple loci (Charlesworth, 2016; Charlesworth & Charlesworth, 1975; Kirkpatrick & Barrett, 2015; Yeaman, 2013). However, it remains to be determined whether chromosomal variants in *T. cristinae* indeed contain multiple genes controlling colour or pattern, which mutations are causal, and whether additional traits adaptive to different plant parts map to the same genomic region.

4.2 | Maintenance of polymorphisms through time

Our results are consistent with chromosomal variants in *T. cristinae* having been maintained by balancing selection through the recent and distant history of the species. LD is expected to decay or build up over tens to thousands of generations by recombination, gene flow or genetic drift (Garrigan & Hedrick, 2003; Hedrick, 2012).

Increased LD in high-differentiation scaffolds relative to the genomic background (Figures 4 and 5b; Fig. S13b) indicates that recombination or gene flow was reduced, or drift increased in this genomic region. This is consistent with balancing selection acting during the recent history of populations, but can also be caused by a selectively neutral inversion polymorphism. However, in this latter case, we would not expect the polymorphism to be present in all populations, which we observed here (Figure 2; Table S1). Tajima's D is affected by mutation and selection, where a signal of balancing selection might require many thousands or millions of generations to be generated or lost, although the statistic can also be influenced by more recent population dynamics such as genetic drift and population structure (Garrigan & Hedrick, 2003; Hedrick, 2012; Simonsen et al., 1995). As recent population dynamics will affect Tajima's D genomewide (i.e., not restricted to high-differentiation scaffolds), we conclude that past balancing selection enabling the accumulation of independent substitutions within chromosomal variants is more likely (Figure 5c; Figs S7 and S13c). Further, the particular patterns in π , D_{xy} and Z_g along the genome between colour variants are consistent with an old inversion polymorphism (Guerrero et al., 2012; Navarro et al., 2000; Peischl et al., 2013) or long-term multilocus balancing selection (Kelly & Wade, 2000; Navarro & Barton, 2002; Nordborg & Innan, 2003; Storz & Kelly, 2008).

The long-term maintenance of polymorphisms is considered to be probably unusual (Asthana et al., 2005; Charlesworth, 2006; Fijarczyk & Babik, 2015). In *Drosophila melanogaster*, for example, inversion polymorphisms are commonly short-lived and frequently less than hundreds of thousands of years old ($<1 N_e$ generations; Andolfatto, Wall, & Kreitman, 1999; Andolfatto, Depaulis, & Navarro, 2001; Corbett-Detig & Hartl, 2012). Similarly, polymorphic inversions in *Anopheles gambiae* were maintained for $<11,000$ years ($<2.7 N_e$ generations; White et al., 2007, 2009). However, polymorphisms have also been shown to persist for millions of years in some species, such as *Drosophila pseudoobscura* (up to 2 Ma; Schaeffer, 2008; Wallace et al., 2013), the ruff (*Philomachus pugnax*: 3.8 Ma; Lamichhaney et al., 2016) or in the form of sex chromosomes (Charlesworth, 2016), and can even be shared across species boundaries (Leffler et al., 2013; Novikova et al., 2016; White et al., 2009; Wiuf, Zhao, Innan, & Nordborg, 2004). Our results for *T. cristinae* are compatible with the examples for old polymorphisms and indicate that the forces of balancing selection have likely been strong and continuous over time to prevent the loss of this variation. As several *Timema* species related to *T. cristinae* are polymorphic for colour, it will be interesting to test in future work whether colour alleles pre-date speciation events. Alternatively, polymorphisms can be acquired by introgression from a related taxon (e.g., Besansky et al., 2003; Feder et al., 2003), which can falsely suggest their long-term maintenance within a species. We regard recent introgression as unlikely given that speciation events in the genus occurred millions of generations ago and *T. cristinae* being geographically isolated from other *Timema* species (Law & Crespi, 2002; Riesch et al., 2017), although we

cannot exclude introgression from a now-extinct species (e.g., Tuttle et al., 2016).

4.3 | Mechanisms of the maintenance of polymorphisms

We detected a pronounced excess of heterokaryotypes within several populations that cannot easily be explained by negative assortative mating or universal mating advantage of the melanistic morph alone (Figure 5a; Table S1; Figs S13a, S21 and S22). Instead, heterokaryotypes might have a fitness advantage over homokaryotypes, for example, because of selective trade-offs (e.g., Johnston et al., 2013), associative overdominance (Charlesworth & Willis, 2009; Pamilo & Palsson, 1998), improved crypsis resulting from differences in body colour compared to homokaryotypes (Tables S8 and S9; Fig. S23) or a combination of these processes.

Although heterozygote advantage can constitute a simple mechanism of balancing selection, it remains controversial whether it maintains variants over extended periods of time (Charlesworth & Charlesworth, 2010; Clarke, 1979; De Boer, Borghans, van Boven, Kesmir, & Weissing, 2004; Hedrick, 2012; Sellis, Callahan, Petrov, & Messer, 2011; Spurgin & Richardson, 2010). Indeed, few empirical examples exist where heterozygote advantage selection is considered to maintain polymorphisms (reviewed by Gemmill & Slate, 2006; Hedrick, 2006, 2011, 2012), often due to a mutant allele that confers improved fitness but is lethal in homozygotes. However, such a polymorphism is expected to be short-lived as it will be lost once a new allele evolves that is not associated with a fitness cost (Charlesworth & Charlesworth, 2010; Clarke, 1979; Hedrick, 2012).

Similarly, heterokaryotype excess involving highly differentiated chromosomal variants often includes lethality of one homokaryotype, where the polymorphism is commonly maintained by negative assortative mating (e.g., Kuepper et al., 2016; Lamichhaney et al., 2016; Tuttle et al., 2016; Wang et al., 2013). However in *T. cristinae*, despite the presumably long divergence time between colour variants, both homokaryotypes are represented. The system might however eventually transition to the more commonly observed situation described above, for example, through the accumulation of recessive deleterious mutations in the rare variant.

In addition to heterokaryotype excess, additional processes of balancing selection probably contribute to the maintenance of colour polymorphism in *T. cristinae*. In particular, the availability of micro-niches (i.e., stems and leaves) on each host plant likely support the maintenance of two colour morphs (Levene, 1953; Nagylaki, 2009). Universal mating advantage of the melanistic morph might further prevent the stochastic or selective loss of the less common melanistic variant (Fig. S22). Thus, although apostatic selection and predator wariness are often considered important mechanisms maintaining polymorphisms in species that use colour traits as protection against predation (Allen, 1988; Bond, 2007; Clarke, 1979; Mappes et al., 2005; Wellenreuther et al., 2014), our results suggest that the long-term maintenance of the cryptic polymorphism within *T. cristinae*

populations can instead be driven by several other, collectively acting forms of balancing selection.

4.4 | Implications for adaptation and speciation

Polymorphisms might also be lost through speciation, as divergent selection or reduced recombination between distinct chromosomal variants can drive the evolution of reproductive isolation (Butlin, 2005; Charron, Leducq, & Landry, 2014; Coyne & Orr, 1998; Faria & Navarro, 2010; Hugall & Stuart-Fox, 2012; Kirkpatrick & Barton, 2006; Navarro & Barton, 2003; Schluter & Conte, 2009). The fine-scaled, temporally and spatially highly heterogeneous habitats of *T. cristinae*, however, might instead promote the long-term maintenance of polymorphisms (Gray & McKinnon, 2007; Gulisija & Kim, 2015; Svardal, Rueffler, & Hermisson, 2015), perhaps amplified by small local population sizes that can further delay speciation (Claessen, Andersson, Persson, & de Roos, 2008). Reproductively isolated small populations, if they arise, might also not persist as they are expected to rapidly accumulate deleterious mutations (Lynch, Conery, & Burger, 1995) and are less likely to hold the phenotypic variation necessary to withstand rapidly changing selection pressures (Forsman & Wennersten, 2016; Nei, Maruyama, & Chakraborty, 1975).

In conclusion, our work indicates that several interacting mechanisms of balancing selection may maintain adaptive polymorphisms over extended periods of time, despite individual mechanisms often being regarded to maintain variation only short term. It remains to be determined whether and how often processes of balancing selection other than apostatic selection or predator wariness, as suggested by our work, are important drivers in maintaining cryptic colour polymorphisms in other species. Finally, the melanistic versus green colour morphs of *T. cristinae* illustrate how long-term maintenance of adaptive polymorphisms in micro-niches might constitute an alternative evolutionary outcome to speciation, particularly in fine-scaled heterogeneous environments (e.g., Gray & McKinnon, 2007). Most broadly, our results show how population genomics can yield powerful insights into evolutionary processes and dynamics when combined with ecological data, ideally from multiple traits and across the species range.

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DATA ACCESSIBILITY

The genetic data from population N1 has been deposited in the NCBI Short Read Archive (BioProject PRJNA386212). Phenotypic and processed genetic data used for analysis as well as simulation code have been archived in Dryad (<https://doi.org/10.5061/dryad.jt644>).

AUTHOR CONTRIBUTIONS

D.L., K.L. and P.N. conceived the project. D.L., V.S.C., R.V. and S.R.D. performed data analyses. T.E.F. and R.R. collected data. Z.G. contributed to computer code and discussions. D.L. and P.N. wrote the manuscript, and all authors contributed to further writing and revisions.

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