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HETEROGENEOUS GENOMIC DIFFERENTIATION BETWEEN WALKING-STICK ECOTYPES: "ISOLATION BY ADAPTATION" AND MULTIPLE ROLES FOR DIVERGENT SELECTION

Patrik Nosil,[1,2] Scott P. Egan,[3,4] and Daniel J. Funk[3,5]

[1]*Department of Zoology, University of British Columbia, Vancouver, B.C., Canada*

[2]*E-mail: pnosil@zoology.ubc.ca*

[3]*Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee 37235*

[4]*E-mail: scott.p.egan@vanderbilt.edu*

[5]*E-mail: daniel.j.funk@vanderbilt.edu*

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Genetic differentiation can be highly variable across the genome. For example, loci under divergent selection and those tightly linked to them may exhibit elevated differentiation compared to neutral regions. These represent "outlier loci" whose differentiation exceeds neutral expectations. Adaptive divergence can also increase genome-wide differentiation by promoting general barriers to neutral gene flow, thereby facilitating genomic divergence via genetic drift. This latter process can yield a positive correlation between adaptive phenotypic divergence and neutral genetic differentiation (described here as "isolation-by-adaptation"). Here, we examine both these processes by combining an AFLP genome scan of two host plant ecotypes of *Timema cristinae* walking-sticks with existing data on adaptive phenotypic divergence and ecological speciation in these insects. We found that about 8% of loci are outliers in multiple population comparisons. Replicated comparisons between population-pairs using the same versus different host species revealed that 1–2% of loci are subject to host-related selection specifically. Locus-specific analyses revealed that up to 10% of putatively neutral (nonoutlier) AFLP loci exhibit significant isolation-by-adaptation. Our results suggest that selection may affect differentiation directly, via linkage, or by facilitating genetic drift. They thus illustrate the varied and sometimes nonintuitive contributions of selection to heterogeneous genomic differentiation.

KEY WORDS: **AFLPs, differential gene exchange, ecological speciation, genome scan, natural selection, speciation, *Timema* stick insects.**

Ecological divergence between populations often generates divergent natural selection, resulting in "ecological speciation" when divergent selection drives the evolution of reproductive isolation (Muller 1942; Mayr 1947, 1963; Schluter and Nagel 1995; Funk 1998; Schluter 2000). In recent years, progress in understanding ecological speciation has derived from analyses showing how ecological divergence and key phenotypic traits under selection affect reproductive isolation (Feder et al. 1994; Funk 1998; Via 1999; Rundle et al. 2000; Jiggins et al. 2001; Funk et al. 2002; Bradshaw and Schemske 2003; Rundle and Nosil 2005 for review). Moreover, the taxonomic generality of ecological speciation has received support (Funk et al. 2006) and specific processes such as

resource competition and predation are now known to be involved (Mallet and Barton 1989; Schluter 1994; Rundle et al. 2003; Vamosi 2005; Nosil and Crespi 2006a). Some outstanding questions concern the genetic basis of ecological speciation (Coyne and Orr 2004). For example, how many and what types of genes are associated with adaptation and reproductive isolation, what are their linkage relationships, and how does selection affect different parts of the genome? The most comprehensive understanding of ecological speciation should derive from a combination of these phenotypic and genetic approaches.

The genetics of speciation can be addressed using many approaches, including quantitative trait locus (QTL) mapping, candidate genes, and multilocus genome scans (Nielsen 2005; Vasemagi and Primmer 2005; Hedrick 2006; Noor and Feder 2006; Stinchcombe and Hoekstra 2007). Candidate gene and mapping approaches have identified specific genes controlling phenotypic traits under selection (Bradshaw and Schemske 2003; Colosimo et al. 2005; Hoekstra et al. 2006), determined whether such genes exhibit physical linkage (Hawthorne and Via 2001; Noor et al. 2001; Rieseberg 2001; Feder et al. 2003), and tested whether parallel evolution involves the same or different sets of genes (Nachman et al. 2003; Joron et al. 2006). These methods are powerful, but require a priori knowledge about the function of a gene or genetic crosses, combined with information on the traits under selection.

A different, but complimentary, approach involves screening many individuals for numerous molecular markers in a "genome scan." These scans allow the signature of selection to be detected at the genetic level through the identification of "outlier loci" that show unusually high levels of genetic differentiation between populations. Such loci may be subject to divergent selection, either directly or through tight physical linkage with selected genes (Bowcock et al. 1991; Beaumont and Nichols 1996; Black et al. 2001; Schlötterer 2002; Luikart et al. 2003; Beaumont and Balding 2004; Beaumont 2005; Nielsen 2005; Stinchcombe and Hoekstra 2007). Genome scans do not elucidate the genetic basis of phenotypic traits, but have alternative goals. By adopting population-level genomic approaches, they can quantify the minimal number of selected gene regions and evaluate evolutionary patterns at selected (i.e., outlier) versus neutral loci. They therefore allow investigation of how and why divergence varies across the genome. Although still relatively modest in number, empirical genome scans are accumulating and generally report that several percent of loci behave as outliers (2–10%, Stinchcombe and Hoekstra 2007 for review; Wilding et al. 2001; Emelianov et al. 2003; Campbell and Bernatchez 2004; Scotti-Saintagne et al. 2004; Acheré et al. 2005; Turner et al. 2005; Vasemagi et al. 2005; Bonin et al. 2006; Murray and Hare 2006; Savolainen et al. 2006; Yatabe et al. 2007). These findings imply that divergence during speciation may involve only a few key genomic regions and are consistent with the concept of a "porous genome" in which genetic divergence is highly variable across loci, with regions under selection and those tightly linked to them exhibiting stronger differentiation than neutral regions (Via 2001; Wu 2001; Vines et al. 2003; Gavrilets and Vose 2005).

Another approach to understanding genetic divergence during speciation examines differentiation at putatively neutral loci in relation to the degree of adaptive phenotypic divergence. Adaptive divergence can cause the evolution of reproductive barriers (i.e., ecological speciation), resulting in a general barrier to even neutral gene flow (for theory see Barton and Bengtsson 1986; Charlesworth et al. 1997; Pialek and Barton 1997; Gavrilets and Cruzan 1998; Gavrilets 2004, p. 147–148 for summary). For example, when populations living in different habitats evolve adaptive preferences for their native habitat, limited dispersal between habitats reduces gene flow between them. Likewise, adaptive trait divergence between populations can result in selection against migrants that disperse into habitats to which they are maladapted, thus reducing gene flow between divergently adapted populations (Nosil et al. 2005 for review). We stress that the general barriers mechanism invokes a role for divergent selection and adaptive divergence in reducing gene flow, but does not require reproductive barriers to be ecological per se. Adaptive divergence can result in the incidental evolution of all components of reproductive isolation, including "nonecological" barriers such as intrinsic hybrid inviability (Muller 1940; Dobzhanksy 1951; Orr 1995; Gavrilets 2004; Dettman et al. 2007).

General barriers reduce the homogenizing effects of gene flow across the genome, thereby allowing neutral regions to diverge via genetic drift. If neutral gene flow decreases as adaptive divergence increases, the resulting increase in drift may result in a positive correlation between the degree of adaptive phenotypic divergence and differentiation at neutral loci (e.g., nonoutliers in a genome scan). This pattern is analogous to isolation-by-distance (IBD), but arises from increased opportunity for genetic differentiation as adaptive, rather than geographic, distance increases. Thus, we hereafter refer to positive correlations between adaptive phenotypic and neutral genetic population divergence, independent from geographic distance, as "isolation-by-adaptation" (IBA). IBA should be detectable on the spatial scale at which gene flow occurs. Several studies have now reported this pattern (MacCullum et al. 1998; Lu and Bernatchez 1999; Cooper 2000; Ogden and Thorpe 2002; Rocha et al. 2005; Grahame et al. 2006; Parchman et al. 2006; Pilot et al. 2006), but others report high gene flow in the face of adaptive divergence (Hendry and Taylor 2004; Smith et al. 2005; Crispo et al. 2006; Yatabe et al. 2007). The causes of these variable outcomes remain unclear. Importantly, not all neutral loci will necessarily exhibit IBA, because neutral differentiation at a given locus is dependent on the stochastic nature of genetic drift. Thus the proportion of neutral loci exhibiting IBA should vary according to factors that affect drift such as levels

of gene flow, effective population size, and time since divergence. To date, studies demonstrating IBA have done so using a small number of loci or by pooling across loci. Thus, the proportion of the genome exhibiting IBA has not previously been evaluated.

Nonoutlier loci might also become associated with adaptive divergence if the effects of selection extend beyond selected regions, not only to tightly physically linked regions (which are likely to be detected as outliers), but also to neutral "weakly linked" regions that are farther removed on the chromosome (Charlesworth et al. 1997; Nielsen 2005). Such weakly linked regions might not be detected as outliers, but are still (more moderately) affected by divergent selection and thus can exhibit IBA. Under this scenario, IBA is expected only for loci close enough to selected regions that their evolution is not completely neutral. Notably, the effects of selection can extend far along a chromosome (Charlesworth et al. 1997). Furthermore, the effects of weak linkage and general barriers might combine to produce IBA. In such cases, the spatial scale of gene flow can help evaluate the relative roles of these two mechanisms. For example, when gene flow is absent due to physical barriers, divergence proceeds entirely by selection and drift and IBA should still be detectable for loci affected by weak linkage. This contrasts with the general barriers scenario in which IBA is not expected in the absence of gene flow, because of the equal opportunity for ecologically similar and ecologically divergent populations to diverge via drift. Under both mechanisms, divergent selection plays a role in the differentiation of functionally neutral loci that are not tightly linked to genes under selection.

Questions remain concerning the proportion of loci affected by each of the processes described above, and how the processes interact (Nielsen 2005; Vasemagi and Primmer 2005; Hedrick 2006; Noor and Feder 2006; Stinchcombe and Hoekstra 2007), because past genome scans have almost never tested for IBA (but see Grahame et al. 2006). A powerful application of the genome scan approach uses replicated comparisons of different types of population pairs (e.g., those that differ vs. those that are similar for a specific ecological variable), but this comparative approach has rarely been applied. This approach is powerful because parallel divergence across multiple population pairs is unlikely to arise via type I error, genetic drift, or mutation rate variation, and because it allows the selective causes of outlier loci to be inferred (Campbell and Bernatchez 2004; Bonin et al. 2006, 2007; S. P. Egan, P. Nosil, and D. J. Funk, unpubl. ms.). To take advantage of the insights provided by an integrated approach, the present study thus combines a comparative genome scan with tests for IBA. Specifically, we examine genome-wide differentiation at hundreds of amplified fragment length polymorphism (AFLP) loci in host plant ecotypes of *Timema cristinae* walking-stick insects. Past phenotypic and experimental data indicate that divergent host adaptation has driven the evolution of reproductive isolation between these ecotypes (Nosil 2007 for review). By examining genomic divergence in these insects we exploit the opportunity to combine ecological, phenotypic, and genetic data to study speciation.

Our design uses replicated comparisons between pairs of populations adapted to different host plant species (different-host pairs) versus population pairs using the same host species (same-host pairs), allowing us to identify loci affected by host-related selection. Thus, this study estimates the specific contribution of host adaptation to genomic differentiation. Finally, and most novel, we use the phenotypic data available from these populations to construct quantitative indices of adaptive divergence between populations pairs. By applying locus-specific analyses of the association of these indices with genetic divergence of nonoutlier loci, we estimate the proportion of the neutral genome that exhibits IBA. Our overall results are consistent with the conclusion that host-related selection plays multiple roles in generating heterogeneous differentiation across the genome. This may occur both through its effect on selected genes and those linked to them, and via reductions in neutral gene flow that facilitate divergence through drift.

Study System

GENERAL DESCRIPTION

Timema walking-sticks are wingless insects inhabiting the chaparral of southwestern North America (Vickery 1993; Crespi and Sandoval 2000). Individuals feed and mate exclusively on the host plants upon which they live. We focus here on *T. cristinae*, which uses two host plant species (*Adenostoma fasciculatum*: Rosaceae and *Ceanothus spinosus*: Rhamnaceae). *Timema cristinae* is composed of two ecotypes (the "*Ceanothus* ecotype" and the "*Adenostoma* ecotype"), which are defined by the host species on which they are found (Nosil 2007 for details).

We define a "population" as all of the walking-sticks collected within a homogenous patch of a single host species (Nosil et al. 2002, 2003, Nosil 2007). Patches of the two host species used by *T. cristinae* are often distributed in adjacent patches that are in geographic contact with one another. We refer to insect populations associated with such patches as "parapatric" (Nosil et al. 2003). Other host patches are separated from patches of the alternative host by distances > 50 times the *Timema* 12 m per-generation migration distance (Sandoval 1993, 2000). Insect populations in such geographically separated patches are termed "allopatric." Pairs of populations on the same host are considered ecologically similar and pairs of populations on different hosts are considered ecologically divergent. Comparisons between these "same-host pairs" and "different-host pairs" can isolate the role of host-related selection in population divergence (Funk 1998; Funk et al. 2002; Funk and Nosil 2007). For example, loci that are outliers in a genome scan only in different-host pairs are candidates for divergence under

host-related selection. Conversely, loci that are outliers between same-host pairs might represent adaptation to host-independent selection (e.g., variation in climate).

The *T. cristinae* ecotypes are divergently adapted to their different hosts. For example, they exhibit genetic divergence in a suite of adaptive morphological traits, and population divergence in these traits has evolved via host-specific selection for crypsis from visual predators (Sandoval 1994a, b; Nosil et al. 2002; Nosil 2004; Nosil and Crespi 2004, 2006a). The ecotypes also exhibit the evolution of reproductive isolation as a consequence of divergent host adaptation (Nosil 2007 for review). Specifically, multiple forms of reproductive isolation are stronger between different-host pairs than between same-host pairs (Nosil et al. 2002, 2003, 2005, 2006a, b, 2007; Nosil 2004; Nosil and Crespi 2006b). In contrast, different-host pairs exhibit no intrinsic F1 hybrid egg inviability (Nosil et al. 2007), no physiological trade-offs in host use (Sandoval and Nosil 2005), and incomplete reproductive isolation (Nosil 2007). Thus, these ecotypes represent stages of divergence prior to the completion of ecological speciation.

SELECTION/GENE FLOW BALANCE AND EXPECTED LEVELS OF ADAPTIVE DIVERGENCE

Morphological and molecular evidence indicate geographically associated patterns of gene flow. For example, morphological and mitochondrial DNA (mtDNA) divergence is consistently and significantly greater between allopatric populations on different hosts than between adjacent, parapatric populations (Sandoval 1994a; Nosil et al. 2003; Nosil and Crespi 2004). This pattern is a classic signature of gene flow (Coyne and Orr 2004), and coalescent-based estimates of mtDNA gene flow ($= m$, the migration rate) into parapatric populations range from 0.001 to 0.232 among populations (mean = 0.043, Nosil et al. 2003). This pattern of greater genetic divergence in allopatry does not rule out the possibility that gene flow into allopatric populations occurs, but indicates that it occurs to a lesser degree than gene flow into parapatric populations. Indeed, maladaptive phenotypes are maintained in allopatric populations, despite strong selection against them, and such phenotypes can even increase in frequency within and among generations, arguing for some recurrent gene flow into allopatry (Bolnick and Nosil, in press).

Past studies have shown that variation in the degree of adaptive divergence between pairs of *T. cristinae* populations (including those examined here) can be reliably predicted by a balance between host-related selection and gene flow (Sandoval 1994a; Nosil et al. 2003; Nosil 2004; Nosil and Crespi 2004). The greatest adaptive divergence is observed when two allopatric populations use different hosts, because divergent selection acts and gene flow is minimal. The weakest adaptive divergence is observed when two allopatric populations use the same host, because divergent selection is lacking. Intermediate levels of divergence occur in comparisons involving parapatric populations. However, even among these comparisons variation can be predicted. For example, a pair comprised of two parapatric populations generally exhibits weaker divergence than a different-host pair in which one population is parapatric and the other is allopatric, because in the former case the adaptation of both populations (rather than just one) is diminished by gene flow. Figure 1 depicts the study populations and expected patterns of adaptive divergence.

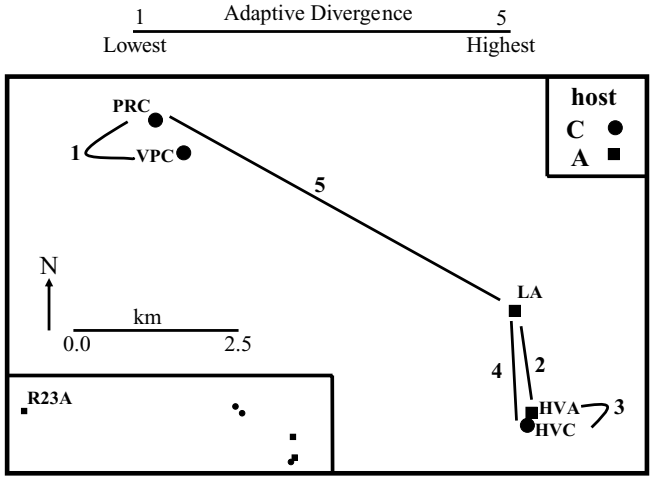

Figure 1. Map of the study populations and a schematic description of expected levels of adaptive divergence between pairs of populations, under the assumption of divergence under a balance between host-associated divergent selection and gene flow. Empirical evidence of such a balance in the *T. cristinae* system is presented in the text. The greatest adaptive divergence occurs when two allopatric populations use different hosts, because divergent selection occurs and gene flow is low (comparison 5). The weakest adaptive divergence occurs when two allopatric population use the same host, because divergent selection is lacking (comparison 1). Intermediate levels of divergence occur in comparisons involving a parapatric population (comparisons 2–4). Of these, comparison 2 has the weakest divergence because both populations use *Adenostoma*. Nonetheless, some genetic divergence is expected because HVA receives alleles conferring adaptation to *Ceanothus* via gene flow from HVC. Comparison 3 is expected to have weaker divergence than comparison 4 because it involves two populations that incur gene flow (HVA and HVC), rather than just one (HVC).

Materials and Methods

STUDY POPULATIONS

We studied one parapatric population pair and two allopatric populations of each host (Fig. 1). Our six study populations yielded 15 pairwise population comparisons that varied in ecological context (nine different-host pairs and six same-host pairs), and in geographic distance. Our selection of populations thus allowed comparisons across a range of evolutionary divergence. Specimens

Table 1. Description of the study populations. The number of individuals genotyped for AFLP markers from each population is given in parentheses to the right of the population name. C = *Ceanothus*. A = *Adenostoma*. % C refers to the percent of the sample site occupied by the host *Ceanothus*.% pick C (*n*) = percent of individuals within a population picking *Ceanothus* over *Adenostoma* as a host in preference experiments (sample size).% striped (*n*) = percent of individuals within a population exhibiting the presence of the striped color pattern (sample size). Body Bright. (*n*) = mean body brightness (sample size). Stripe Bright. = mean stripe brightness (sample size as for body brightness). Baseline information about population variability is provided in the online Supplementary Material.

Pop. (*n*)	Host	Latitude	Longitude	% C	% pick C (*n*)	% striped (*n*)	Body Bright. (*n*)	Stripe Bright.
1. PRC (31)	C	34 32.03	119 51.48	100	93 (65)	1 (1091)	44 (62)	48
2. VPC (32)	C	34 31.89	119 50.56	100	91 (174)	9 (633)	47 (13)	46
3. HVC (31)	C	34 29.42	119 47.16	33	79 (29)	61 (144)	46 (16)	54
4. HVA (34)	A	34 29.31	119 47.19	33	78 (98)	85 (1279)	40 (65)	52
5. LA (32)	A	34 30.52	119 47.75	0	63 (139)	86 (615)	38 (25)	50
6. R23A (32)	A	34 31.60	120 04.34	0	52 (56)	94 (395)	37 (68)	52

were collected near Santa Barbara, California by using sweep nets to randomly sample throughout host patches. A total of 192 field-collected individuals ($n > 30$ in all populations) were selected for AFLP genotyping (see Table 1 for population-specific information and Table 2 for population differentiation).

AFLP PROTOCOLS

Whole genomic DNA was extracted using a few legs per specimen, following DNAeasy Animal Extraction Kits (Qiagen, Valencia, CA). To generate AFLP data following Vos et al. (1995), we used AFLP Core Reagent Kits (Invitrogen, Carlsbad, CA). After the preselective amplification, eight selective amplification primer combinations were used to generate PCR products that were then purified using Sephadex (GE Healthcare, Uppsala, Sweden) (for information on primers see online Supplementary Material). Products were run on 6% polyacrylamide gels in the Department of Biological Sciences at Vanderbilt University using an MJ Base Station Automated Sequencer (MJ Research, Waltham, MA). Fragment analysis was completed using Cartographer software associated with the Base Station. Loci between 65 and 550 bp were identified manually and locus ranges were set within Cartographer. Presence versus absence of peaks for these loci was first called automatically within Cartographer according to a noise threshold set by the program within each lane, with alleles exhibiting fluorescent signal above the threshold called as present. Most importantly, all loci for all individuals were then visually inspected

Table 2. Divergence between the 15 pairs of populations of *Timema cristinae*. Provided is the type of host comparison (DH = different-host pair, SH = same-host pair), the geographic distance between populations (GeoD), percent mtDNA sequence divergence, the number of outliers at the 95 and 99 quantiles, the number of loci analyzed for each population pair (no. of loci = number of the 534 total loci that were polymorphic for a particular population pair), F_{ST} between populations for each of four classes of loci (Host comp. = Host comparison; Neut. = Neutral, DH = DH-Specific Outliers), and trimmed F_{ST} estimated using different trimming thresholds in Dfdist (30%, 20%, 10%).

Pair	Host comp.	GeoD (km)	% mtDNA	# 95% (99%) outliers	No. of loci	F_{ST} Total	F_{ST} Neut.	F_{ST} Other	F_{ST} DH	F_{ST} 30% trim	F_{ST} 20% trim	F_{ST} 10% trim
1. PRC vs. HVA	DH	8.27	2.9	3 (0)	407	0.048	0.042	0.068	0.091	0.029	0.033	0.040
2. PRC vs. LA	DH	6.35	3.5	10 (5)	426	0.057	0.030	0.134	0.204	0.033	0.036	0.043
3. PRC vs. R23A	DH	19.65	4.5	6 (1)	451	0.183	0.118	0.404	0.205	0.138	0.149	0.163
4. VPC vs. HVA	DH	7.02	2.9	11 (5)	418	0.129	0.065	0.300	0.439	0.081	0.092	0.107
5. VPC vs. LA	DH	4.99	3.6	7 (2)	426	0.089	0.051	0.186	0.279	0.053	0.060	0.072
6. VPC vs. R23A	DH	21.05	4.5	13 (6)	418	0.084	0.037	0.185	0.360	0.043	0.052	0.062
7. HVC vs. HVA	DH	0.21	2.5	18 (9)	399	0.063	0.023	0.194	0.234	0.022	0.028	0.043
8. HVC vs. LA	DH	2.23	1.7	15 (5)	413	0.024	0.010	0.068	0.098	0.004	0.008	0.014
9. HVC vs. R23A	DH	26.55	4.0	17 (6)	436	0.082	0.032	0.223	0.218	0.042	0.050	0.063
10. PRC vs. VPC	SH	1.43	1.9	4 (1)	414	0.199	0.132	0.362	0.528	0.148	0.158	0.180
11. PRC vs. HVC	SH	8.18	3.6	8 (2)	422	0.119	0.074	0.258	0.330	0.075	0.083	0.096
12. VPC vs. HVC	SH	6.92	3.6	15 (7)	405	0.039	0.016	0.089	0.031	0.015	0.019	0.027
13. HVA vs. LA	SH	2.40	2.7	21 (5)	413	0.022	0.005	0.075	0.039	0.004	0.009	0.015
14. HVA vs. R23A	SH	26.53	4.0	15 (8)	443	0.137	0.072	0.363	0.152	0.091	0.099	0.113
15. LA vs. R23A	SH	25.41	3.4	14 (7)	454	0.105	0.057	0.271	0.121	0.065	0.073	0.083

and manually adjusted (hand-called) by PN. While hand-calling, samples that had a weak or noisy signal were noted and rerun. All scoring was done blind to population of origin.

REPEATABILITY ESTIMATES

To ensure high repeatability of analyzed AFLP loci, every individual was genotyped twice for all primer pairs. Each gel included individuals from both ecotypes and multiple study populations. Replicate samples of the same individual were run on different gels. The repeatability of each individual locus was estimated as one minus the ratio of the total number of differences for that locus to the total number of individuals genotyped (following Bonin et al. 2004; Pompanon et al. 2005). Individual loci that were less than 90% repeatable were excluded from further analysis. The remaining markers were used to estimate the average "per locus genotyping error rate," as the ratio of the total number of differences across all loci to the total number of comparisons across loci (Bonin et al. 2004; Pompanon et al. 2005). This value was <4%.

EMPIRICAL DISTRIBUTION OF F_{ST} AND OUTLIER DETECTION USING SIMULATIONS

Simulations can model expected patterns of population differentiation for neutral loci. Actual loci whose empirically derived levels of differentiation place them at the upper extreme of simulated distributions of differentiation are considered "outlier loci" that are putatively subject to divergent selection (Bowcock et al. 1991; Black et al. 2001; Luikart et al. 2003; Nielsen 2005; Stinchcombe and Hoekstra 2007). Theoretical studies have shown that such divergence-based methods have high power to detect loci subject to divergent selection (Beaumont and Nichols 1996; Beaumont and Balding 2004; Beaumont 2005). To identify outlier loci, we thus adopted the approach of Beaumont and Balding (2004), implemented in the program Dfdist. This comprised three steps, each of which was applied separately to each of the 15 population pairs.

First, the empirical distribution of F_{ST} values among loci was generated using a Bayesian method in which allele frequencies are estimated from the proportion of recessive genotypes in the sample (Zhivotovsky 1999). Second, we estimated the mean F_{ST} value for neutral loci by trimming loci at the extremes of this empirical distribution. Our "trimmed" mean F_{ST} was calculated by removing the highest 30% and lowest 30% of F_{ST} values observed in the empirical dataset; it represents an estimate of the average "neutral" F_{ST} value, uninfluenced by selection on specific loci (Beaumont and Balding 2004). This particular (30%) trimming threshold is recommended by Beaumont and Balding (2004), has been adopted in other genome scan studies (e.g., Bonin et al. 2005), and is used for the focal analyses presented in this study. Nonetheless, to evaluate the robustness of our results to the trimming procedure, we also conducted analyses using alternative thresholds of 20% and 10%. These lower thresholds might be expected to lower estimates of average neutral F_{ST} and thus more conservatively identify outliers. The 20% and 10% thresholds yielded average F_{ST} values that differed only modestly from those observed for the 30% threshold (see Table 2). Moreover, F_{ST} values derived from the different thresholds were highly correlated across the 15 population pairs (all $r > 0.997$, all $P < 0.001$). Additional findings derived from the alternative thresholds are discussed below.

Second, a distribution of simulated F_{ST} values was generated, in which the mean simulated F_{ST} was targeted to match the mean trimmed F_{ST} calculated from the empirical distribution. A hierarchical Bayesian approach was used to compute these F_{ST} values conditional on heterozygosity in a subdivided population under the infinite island model (Wright 1943). We thereby generated 50,000 simulated loci with a mean F_{ST} similar to the trimmed mean F_{ST} (the correlation between mean simulated and mean empirical trimmed F_{ST} across the 15 population pairs, was $r = 0.999$ $P < 0.001$).

Third, the empirical and simulated distributions were compared to identify outliers. From the simulated loci, an outlier threshold was calculated at both the upper 95th and 99th quantiles using a smoothing parameter of 0.04. Loci above these thresholds were considered outliers. Because divergence-based methods have extremely low power to detect stabilizing selection (Beaumont and Nichols 1996; Beaumont and Balding 2004), we did not specifically test for loci with unusually low F_{ST}. These methods also do not specifically consider relaxed selection, a form of weak divergent selection, such that some loci under weak selection may not be detected.

CLASSIFICATION OF OUTLIERS

After outlier detection, loci were categorized according to the types of comparisons in which they were outliers (see Table 3). Loci that were not outliers in any comparison were considered putatively neutral. Host plant use by the study populations, and the replication of outlier behavior across population pairs, was used to further categorize loci. Loci that were outliers in only one pairwise comparison were deemed "nonrepeated outliers." These nonrepeated outliers were further classified into whether they were outliers in a same-host pair and or in a different-host pair. Loci that were outliers in multiple comparisons were denoted "repeated outliers." These repeated outliers were classified according to whether they were outliers in same-host pairs only, "mixed outliers" (i.e., those observed in a mixture of same-host and different-host pairs) that were identified in at least as many same-host as different-host comparisons, mixed outliers that were identified in more different-host than same-host comparisons, or outliers only in different-host pairs. We also noted outliers that were restricted to different-host pairs and observed in multiple independent comparisons, that is, comparisons that did

Table 3. Outlier detection for the 534 AFLP loci at the 95% (99%) quantile. DH = different-host pair. SH = same-host pair.

Outlier distribution	Type of comparison	No. of loci	Class of loci	Possible inferences
Observed in zero comparisons		456	Neutral Loci	-putatively neutral
Observed in one population comparison ("nonrepeated" outliers)	Same-host	17 (10)		- host-independent selection
	Different-host	19 (9)		- host-associated selection
Observed in > 1 population comparison ("repeated" outliers)	Same-host only	1 (0)	Other Outliers	- host-independent selection
	Both, but DH ≤ SH	24 (9)		
	Both, but DH > SH	12 (4)		- host-independent selection or host-associated selection with outliers influenced by gene flow
	Different-host only	5 (1)	DH-Specific Outliers	- host-associated selection

not share a population in common. The categorization scheme was used to make inferences about possible causes of outlier behavior (Table 3).

To further compare loci subject to different evolutionary processes, the analyses of this article especially focus on datasets representing different classes of loci that are hereafter referred to using the following terms: (a) Total Loci = all 534 AFLP loci, (b) Neutral Loci = loci that were not outliers in any comparison, (c) Other Outliers = loci that were outliers in multiple comparisons including same-host pairs, and (d) DH-Specific Outliers = loci that were outliers in multiple different-host comparisons only.

VERIFICATION OF DH-SPECIFIC OUTLIERS

Because of our particular interest in DH-Specific Outliers, we used three additional methods to corroborate their outlier status and host specificity. First, we conducted a "global" Dfdist analysis by pooling individuals across populations using the same host into a single "host population" and then identifying outlier loci in a comparison of the two host populations. Second, for each DH-Specific Outlier we ran a separate logistic regression analysis with genotype (band present versus absent) as the dependent variable and host of origin as the independent variable. A likelihood-ratio (LR) test was used to determine whether host significantly predicted genotype. Results from these analyses should be interpreted with some caution as they pool individuals from different neutral backgrounds. However, they are nonetheless useful for verifying that particular loci represent strong between-host outliers, and the regression approach in particular has been advocated for outlier verification because it does not rely upon a trimming threshold (Bonin et al. 2006, 2007). For example, Dfdist could yield "false positives" in the form of loci that barely exceed the 95% threshold or appear in few comparisons. Such loci might not be identified as host specific in these additional analyses. In contrast, loci that were DH-Specific Outliers in only a few different-host pairs, yet approached the 95% quantile in other such pairs, may have their host-specific status further verified by these analyses. Third, we tested whether loci that were DH-specific Outliers using the conventional 30% trimming threshold remained statistical outliers using the alternative and more conservative 20% and 10% thresholds. These analyses were conducted for all the population pairs in which at least one DH-Specific Outlier had exceeded the 95% quantile in the focal analyses using a 30% threshold.

CAVEATS REGARDING OUTLIER LOCI: MUTATION RATE VARIATION AND TYPE I ERROR

In view of the nature of our study, two issues must be addressed before outlier behavior can be attributed to the effects of divergent selection. One issue is that variation among loci in levels of genetic differentiation could reflect variation in mutation rates, rather than selection (Balloux and Lugon-Moulin 2002; Hedrick 2005; Noor and Feder 2006). Two arguments indicate that mutation rate variation cannot account for our results. First, and most generally, mutation rate is not predicted to strongly affect genetic differentiation for any class of loci when gene flow between populations occurs (Beaumont and Nichols 1996; Balloux and Lugon-Moulin 2002; Hedrick 2005), as is likely in *T. cristinae*. This argument stems from theory indicating that in an island model of migration (Wright 1931), F_{ST} is a decreasing function of N ($m + \mu$), the product of local population size and the sum of migration and mutation rates (Hartl and Clark 1997; Balloux and Lugon-Moulin 2002). So when migration is greater than mutation, as is likely with even very low migration rates, genetic differentiation is dominated by migration, and is thus unlikely to be explained by mutation. Thus, the documented gene flow between *T. cristinae* populations (Sandoval 1994a, b; Nosil et al. 2003; Nosil and Crespi 2004; Bolnick and Nosil, in press) argues against an important role for the effects of mutation rate on F_{ST} and outlier detection in our study. Second, variable mutation rates cannot explain our observation of loci that are highly differentiated only in comparisons of ecologically divergent (i.e., different-host) pairs of populations. In essence, our same-host population pairs act as controls for the potential effects of mutation rate variation on genetic differentiation.

Similar arguments apply to other potentially confounding factors, such as sex-linkage, whereby reduced effective population size of sex-linked loci could increase genetic divergence by drift, resulting in high F_{ST} of sex-linked loci that are not affected by selection. However, as for mutation rate variation, this process is not expected to result in DH-Specific Outliers.

Another issue for outlier detection, reflecting the large number of loci screened in a genome scan, is type I error due to multiple comparisons. For example, within any single pairwise population comparison the number of loci expected to be outliers by random chance (type I error) alone = 0.05 × no. of loci. Fortunately, the Bayesian methods implemented in our study (Beaumont and Balding 2004; p. 979 for details) explicitly address this issue, by using the prior distribution to correct for the potential problem of multiple testing. Thus, outliers detected in our study are unlikely to represent type I error (i.e., false positives).

Moreover, our particular study design further minimizes the chances of detecting outliers that are false positives. It does so by focusing on the identification of loci detected to be outliers in each of multiple pairs of populations, and especially on those detected as outliers in multiple different-host population comparisons only. Repeated outliers are very unlikely to arise via type I error because the probability that type I error can explain a pattern decreases as a function of the repeatability and consistency of that pattern. We thus provide the following equation for calculating the number of outliers expected by type I error alone. This equation is conservative because it assumes that Dfdist does not address type I error (although it does). It also specifically accounts for both the number of loci and the number of pairwise population comparisons. On this point, given 15 total population pairs (including nine different-host pairs) and the evaluation of 534 loci, the number of outlier loci expected to be observed in n different-host comparisons (and no same-host comparisons) is

$$(0.05^n \times 534) \times \frac{\binom{9}{n}}{\binom{15}{n}}. \quad (1)$$

LINKAGE DISEQUILIBRIUM ANALYSES

To make inferences about the genomic distribution of outlier loci, we quantified levels of linkage disequilibrium among loci within each of the populations. Separate analyses were conducted for the Neutral Loci, Other Outliers, and DH-Specific Outliers classes. If outlier loci are physically linked to each other, their linkage disequilibrium is expected to be elevated relative to neutral loci, for example, due to the effects of selective sweeps (Kim and Nielsen 2004). This physical linkage is expected to result in similar levels of disequilibrium within allopatric and parapatric populations (Arnold 1992). In contrast, if linkage disequilibrium is caused by migration between differentiated populations (Kimura 1956; Nei and Li 1973; Kirkpatrick et al. 2002), then disequilibrium should be greater within parapatric populations (Nosil et al. 2006b).

We estimated linkage disequilibrium using two methods, which examine pairs of loci and overall multilocus disequilibrium, respectively. The first approach used the program DIS (Dasmahapatra et al. 2002) to estimate pairwise linkage disequilibria between loci for dominant data (R = the gametic correlation coefficient correcting for variable allele frequencies), using a maximum-likelihood equivalent to that of Hill (1974). This program is limited to 40 loci, so we analyzed the five DH-Specific Outliers, the 37 Other Outliers, and 40 randomly selected Neutral Loci. We used paired t-tests (with population as the unit of replication) to examine whether classes of loci differed significantly in levels of linkage disequilibrium. The second method used LIAN 3.1, which allowed consideration of all loci within a class (Haubold and Hudson 2000). LIAN tests for independent assortment by computing the number of loci at which pairs of individuals differ. From the distribution of these mismatch values, a variance (V_D) is calculated. This is compared to the variance expected for linkage equilibrium (V_E), allowing computation of a standardized index that represents multilocus association (I_A^S). Paired t-tests were used to examine whether classes of loci differed in their association values across populations.

PHYLOGEOGRAPHIC PATTERNS AND GENETIC STRUCTURE

For each class of loci, genetic differentiation (F_{ST}) among populations was calculated using 1000 bootstraps in AFLP-SURV version 1.0 (Vekemans et al. 2002). Then, for each class of loci, 1000 bootstrapped Nei's genetic distance matrices generated by AFLP-SURV were used to construct a neighbor-joining 50% majority rule consensus tree with the programs NEIGHBOR and CONSENSE within PHYLIP 3.6 (Felsenstein 2004). A version of Structure (2.2., Falush et al. 2007) modified for dominant markers was used to perform the Bayesian assignment analysis of Pritchard et al. (2000). This method is conventionally used to determine the number of genetic clusters that best fit the data. However, Structure has many additional uses (Pritchard et al. 2000), and our focus here is on potential genetic differentiation according to ecotype, rather than on determining the number of genetic clusters per se. Thus, we assumed two clusters ($K = 2$) and tested whether those identified by Structure corresponded to the two host plant ecotypes. Separate analyses were run for each class of loci and three separate runs for each class yielded essentially identical results. For each analysis, we implemented a burn-in of 50,000 generations and a Markov Chain of 500,000 generations using the admixture model. For each class of loci, a nested ANOVA tested whether assignment proportions differ between host ecotypes and among populations within ecotypes.

ISOLATION-BY-ADAPTATION

We tested for IBA using indices of adaptive divergence that reflect the balance between selection and gene flow (see also Fig. 1). Specifically, we constructed three indices representing different ecologically adaptive traits: host plant preferences, ecomorphology, and host plant composition (Table 1). Host preference was estimated for each of the six populations using host-choice experiments, where the host choice of individual walking-sticks was recorded as described in Nosil et al. (2006a; total $n = 561$). Data from five of the populations come directly from Nosil et al. (2006a), whereas comparable data for population R23A were obtained for the present study using the same procedures in May 2006 ($n = 56$). Divergence in preference for each population pair was calculated as the difference between populations in the mean percentage of individuals choosing *Ceanothus* over *Adenostoma*.

Ecomorphological divergence was estimated using three morphological traits known to differ between ecotypes and to be under strong host-specific divergent selection for crypsis. These traits are the presence versus absence of a genetically determined dorsal stripe (Sandoval 1994a, b), body brightness, and stripe brightness. The latter two traits are quantitative measures of the brightness of the exterior and central parts of the body respectively, irrespective of the presence/absence of the stripe itself (Nosil and Crespi 2006a for details). Population divergence in stripe frequency was estimated from 4157 individuals sampled across all current study populations from 2001 to 2006 (Nosil 2004, unpubl. data). Nosil and Crespi (2006a) examined 249 specimens from our six populations. From that study, divergence in body brightness was estimated as the absolute value of the difference in mean brightness between a pair of populations. The same was done for divergence in stripe brightness. To reduce redundancy, we calculated a principal components axis using the three traits. The first axis (PC1) explained 60% of the variance among population pairs, loaded each trait strongly and positively (all loadings > 0.60), and was used in all subsequent analyses.

The third index represents differences in host plant composition between sites. Specifically, the percentage of each study site occupied by *Ceanothus* was estimated using aerial photos (as in Sandoval 1994a,b; Nosil et al. 2003). For example, allopatric populations of *Ceanothus* and *Adenostoma* would be assigned values of 100 and 0, respectively. A parapatric population at a site in which each host covered an equal area (i.e., where half the collective area of the site is *Ceanothus*) would be assigned a value of 50. Our index of divergence is the difference between populations in these values (*% Ceanothus* hereafter). Such differences in percent *Ceanothus* were correlated with host preference and morphology in past studies (Nosil and Crespi 2004; Nosil et al. 2006a), raising the issue of redundancy among our three indices. We feel that the use of all three is warranted because they encompass biologically different measures of adaptive divergence and because correlations among them are not overly strong (host preference vs. PC1, $r = 0.44$, $P = 0.11$; host preference vs. *% Ceanothus*, $r = 0.62$, $P = 0.05$; PC1 vs. *% Ceanothus*, $r = 0.79$, $P < 0.05$; Mantel tests). Although these indices do not provide completely independent tests of association between adaptive and genetic divergence, using all three helps evaluate the robustness of our findings.

We used simple and partial Mantel tests (Manly 1997) to examine associations among genetic differentiation, adaptive divergence, and geographic distance. Specifically, simple Mantel tests were used to examine the association between genetic divergence and adaptive divergence and the association between genetic divergence and geographic distance. Partial Mantel tests were used to examine the association between genetic divergence and adaptive divergence controlling for geographic distance (= IBA), and between genetic divergence and geographic distance controlling for adaptive divergence (= IBD). We first conducted analyses in which the measure of genetic divergence was overall F_{ST} across loci within each class. All Mantel tests implemented 1000 randomizations and were conducted using the software Isolation by Distance version 1.52 (Bohonak 2002), which reports one-tailed probabilities. This program calculates r, the correlation between two matrices, which ranges from -1 to $+1$ (Manly 1997; Bohonak 2002). Analyses were conducted using both raw and log-transformed distance matrices to ensure the results were robust to negative estimates of F_{ST} (which were set to 0.0001 before log transformation). Although significance testing using simple Mantel tests is unbiased, the accuracy of partials is a topic of debate (Raufaste and Rousset 2001; Rousset 2002). However, it appears that partial Mantel tests perform well in most conditions (Castellano and Balletto 2002), and particularly when correlations between independent variables are weak or moderate as observed here (Mantel tests against geographic distance: Host preference, $r = 0.59$, $P = 0.03$; Ecomorphology, $r = -0.02$, $P = 0.52$;*% Ceanothus*, $r = -0.07$, $P = 0.60$).

Next, we conducted Mantel tests for each individual locus using locus-specific F_{ST} values from Dfdist. These locus-specific analyses provide two types of additional information: they identify specific loci exhibiting significant IBA and IBD, and they provide distributions of locus-specific correlation coefficients. F_{ST} could not be calculated for some loci for specific population pairs because these loci were monomorphic for that particular pair. Because these loci result in missing data in the distance matrices they were excluded from the Mantel analyses, yielding a total of 209 loci for analysis.

Given that we evaluated 209 loci, roughly 20 significant (10 positive and 10 negative) spurious correlations due to multiple comparisons might be expected at a one-tailed alpha level of 0.05. We used three lines of evidence to evaluate the possible dependence of our results on this statistical artifact. First, if the associations are not spurious, we expected more than 10 positive

significant associations. Second, for correlations due to chance alone we expected significantly positive and significantly negative correlations in equal proportion. Binomial tests were thus used to examine whether more significant positive associations were observed than expected by chance, as predicted by IBA. Third, spurious associations should yield correlation coefficient distributions that do not differ significantly from zero, which we evaluated using one-sample *t*-tests. After we had confidently identified loci exhibiting significant IBA using these approaches, we examined the degree to which these loci represented outliers in the genome scan.

We additionally tested for IBA and IBD using mtDNA sequence variation. These analyses used 413 bp sequences of the cytochrome oxidase I (COI) gene from 33 *T. cristinae* from the six study populations ($n = 5, 5, 3, 5, 6, 9$ from populations 1–6, respectively). Sequences from five of the populations are previously published (Nosil et al. 2002, 2003); sequences for R23A were obtained using the same procedures (Genbank accession numbers EU251503-EU251511). Population divergence in mtDNA was estimated in MEGA version 3.1 (Kumar et al. 2004) as mean pairwise divergence corrected for multiple hits using the Kimura two-parameter model (Kimura 1980). As for AFLP loci, simple and partial Mantel tests evaluated IBA and IBD. Notably, the problem of multiple comparisons does not apply to this single-locus mtDNA analysis.

Results

OUTLIER DETECTION AND CLASSIFICATION

Outliers were detected in all 15 pairwise comparisons at the 95% level, and in all but one population comparison at the 99% level (Fig. 2). In total, 78 of 534 loci were outliers at the 95% level in at least one comparison. The proportion of loci that were outliers within an individual population pair ranged from 0.7% to 5.1%, with a mean of 3.8% (Table 2). Table 3 presents the number of loci corresponding to their various categories. Forty-two of 78 outlier loci (8% of total analyzed loci) were "repeated." Five loci were DH-Specific Outliers (1% of total analyzed loci) and one of these (D47) was an outlier in two statistically independent different-host comparisons. Only one locus was an outlier in multiple comparisons between same-host pairs but not in any different-host pairs. The remaining repeated outliers appeared in a mixture of different-host and same-host pairs.

Further analyses verified the outlier status and host specificity of DH-Specific Outliers. For example, the global Dfdist analysis detected 20 outliers at the 95% quantile. Four of the five DH-Specific Outliers were among these 20 (L80, E10, D47, and G37). Furthermore, genotype at each of these four loci was highly significantly predicted by host of origin in the logistic regression analyses (L80, LR = 12.50, $P < 0.001$; E10, LR = 7.73, $P < 0.01$; D47, LR = 15.96, $P < 0.001$; G37, LR = 24.45, $P < 0.001$; I15, LR = 0.64, $P = 0.43$; all df = 1). Finally, in analyses using alternative trimming thresholds of 20% and 10%, all five DH-Specific Outliers from the focal analyses using the 30% threshold remained statistically significant outliers in every population comparison in which they were originally detected ($P < 0.05$ in all cases using the *P*-value function in Dfdist, with all loci also above the 95% quantile).

OUTLIERS AND TYPE I ERROR

The efficacy of the method of Beaumont and Balding (2004) in correcting for multiple comparisons and dealing with type I error is illustrated by the observation that we examined 534 loci, yet considerably fewer than the random expectation of 27 outliers ($0.05 \times 534 = 27$) were detected in each of the 15 individual population comparisons (Table 2; mean number of outliers per comparison = 11.8, SD = 5.3). In fact, the mean number of outliers detected per comparison was significantly lower than 27 ($t_{14} = 11.01$, $P < 0.001$, one-sample *t*-test). Probability calculations confirm that more DH-Specific Outliers were detected than expected via type I error alone, even in the absence of Dfdist's correction for multiple comparisons. Following equation (1), we expect less than a single locus (0.8 loci) to be an outlier in two different-host comparisons, but no same-host comparisons (i.e., $n = 2$). In contrast, we detected five loci exhibiting such behavior (i.e., the five DH-specific Outliers), significantly more than expected ($P < 0.001$, binomial probability). Likewise, we expect only 0.0003 loci to be outliers in four different-host comparisons and no same-host comparisons (i.e., $n = 4$). However, we detected such a locus, an observation highly unlikely to be due to type I error ($P < 0.0001$, binomial probability). Thus, even adopting the very conservative stance that the methods of Beaumont and Balding (2004) do not effectively deal with multiple comparisons, probability statements demonstrate a clear excess of DH-Specific Outliers among our assayed loci, relative to the number expected by type I error alone.

LINKAGE DISEQUILIBRIUM

In the DIS analyses, mean pairwise disequilibrium was low, usually approximately 0.10 (Fig. 3). Differences between classes of loci in levels of disequilibrium achieved statistical significance for Other Outliers versus Neutral Loci, with higher disequilibrium observed for the former (mean difference = 0.047, $t_5 = 3.022$, $P < 0.05$). The trend for DH-Specific Outliers versus Neutral Loci was nonsignificantly in the same direction, with higher linkage disequilibrium for DH-Specific Outliers within five of the six populations (mean difference = 0.073, $t_5 = 1.36$, $P = 0.23$). The difference between DH-Specific and Other Outliers was nonsignificant and showed no trend (mean difference = 0.027, $t_5 = 0.574$, $P = 0.59$). Generally congruent results were observed in LIAN analyses, where multilocus disequilibrium was weak and the difference

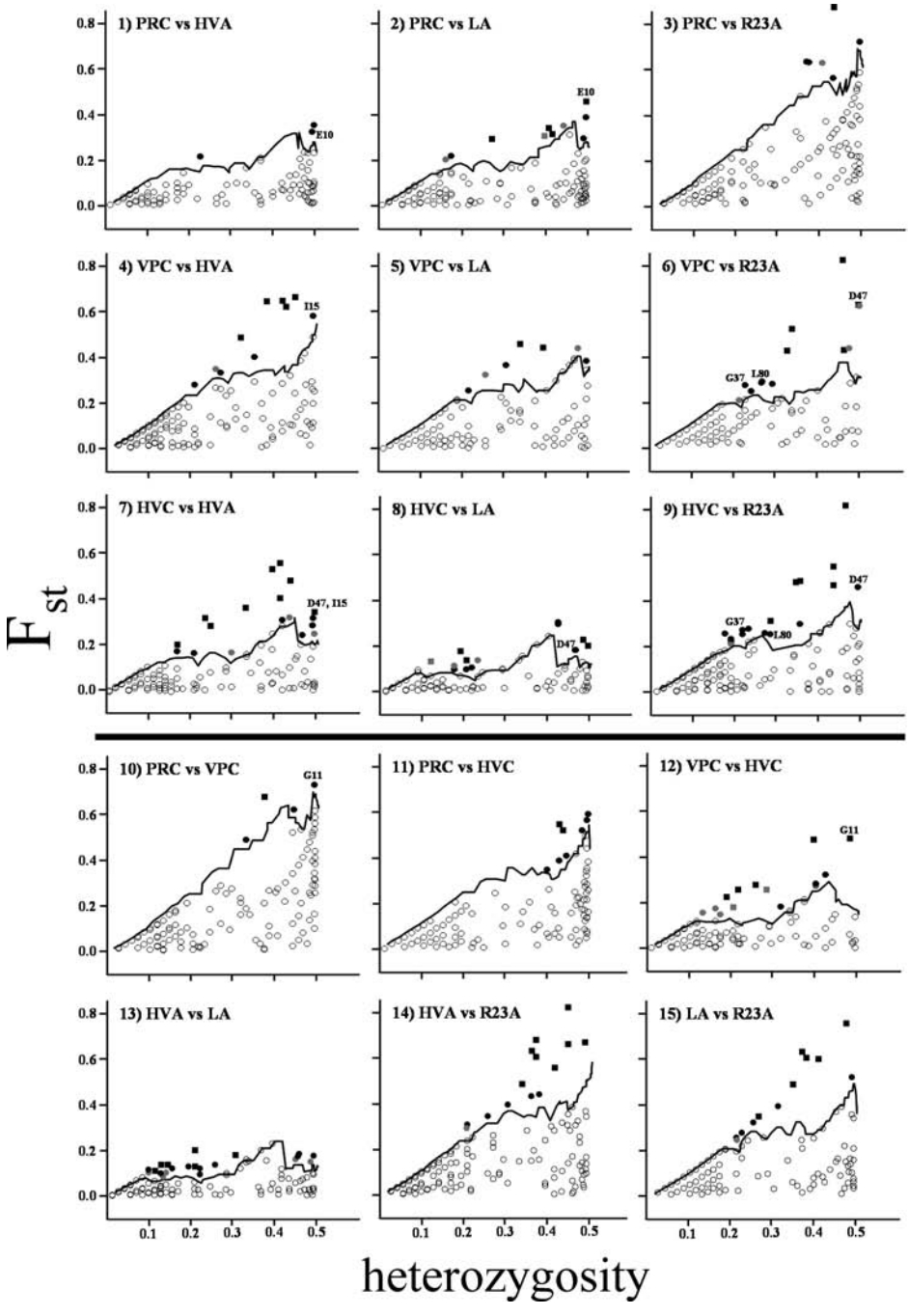

Figure 2. Results of Dfdist analyses. Each of the 15 plots illustrates the empirical distribution of F_{ST} values for individual AFLP loci in relation to a simulated 95th quantile for simulated neutrally evolving loci (solid line). The filled symbols depict loci with F_{ST} values exceeding this quantile, which are "outliers" putatively evolving under divergent selection. "Repeated" outliers that are observed in multiple population comparisons are black, whereas "nonrepeated" outliers are gray. Outliers that also exceed the 99th quantile are squares rather than circles. Different-host pairs are above the thick black line whereas same-host pairs are below it. Repeated outliers that are restricted to different-host pairs or to same-host pairs are labeled. See online Supplementary Material for a detailed description of which loci were outliers within each specific population pair.

between Other Outliers and Neutral Loci was once again significant (Other Outliers vs. Neutral Loci: mean difference = 0.015, t_5 = 2.755, P = 0.040; DH-Specific Outliers vs. Neutral Loci: mean difference = −0.002, t_5 = 0.221, P = 0.834; DH-Specific Outliers vs. Other Outliers: mean difference = −0.0178, t_5 = 2.092, P = 0.091; paired t-tests). Thus, there was little suggestion of stronger linkage disequilibrium within parapatric populations. If anything, outlier loci exhibited stronger linkage disequilibrium than neutral

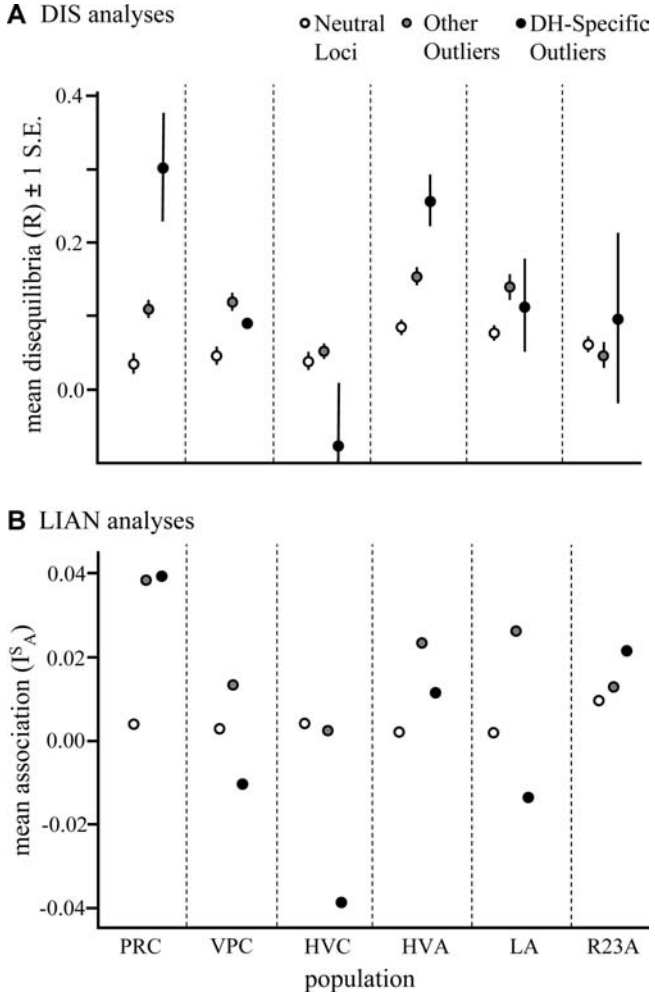

Figure 3. Estimates of linkage disequilibrium among loci within each of the six study populations, for different classes of loci. (A) DIS results showing mean (± 1 SE) pairwise linkage disequilibria. The mean gametic correlation coefficient (*R*) is reported, which corrects for variation in allele frequencies. (B) LIAN results showing a standardized index of association (I_A^S) which measures multilocus disequilibrium. Paired *t*-tests examining differences among classes of loci are reported in the text.

loci independent of geography. These patterns were evident even when the specific 18 outlier loci from the parapatric population pair were considered (linkage disequilibrium was not elevated in parapatry, but was greater than observed for neutral loci in all six populations; $t_5 = 3.55$, $P = 0.016$).

PHYLOGEOGRAPHIC PATTERNS AND GENETIC STRUCTURE

As expected, F_{ST} values were higher for the two outlier classes than for the Total Loci and Neutral Loci classes (Table 2). Neighbor-joining trees reconstructed using different classes of loci exhibited two different topologies (Fig. 4). The Total Loci, Neutral Loci, and Other Outlier topologies were identical and never produced topologies consistent with the union of populations using the same host as sister taxa. The topology based upon DH-Specific Outliers differed from the others and was not consistent with two host-associated monophyletic lineages, although it provided one case highly consistent with the union of two populations using the same host.

Assignment proportions from Structure differed somewhat between ecotypes for Other Outliers (ecotypes $F_{1,192} = 8.48$, $P < 0.01$, populations $F_{4,192} = 100.784$, $P < 0.001$) and for DH-Specific Outliers (ecotypes $F_{1,192} = 2.58$, $P = 0.11$, populations $F_{4,192} = 14.79$, $P < 0.001$). In contrast, there was no evidence that assignment proportions differed between ecotypes for Total Loci (ecotypes $F_{1,192} = 0.01$, $P = 0.98$, populations $F_{4,192} = 49.59$, $P < 0.001$) or Neutral Loci (ecotypes $F_{1,192} = 1.96$, $P = 0.20$, populations $F_{4,192} = 25.74$, $P < 0.001$). Notably, assignment proportions always differed significantly among populations within ecotypes, indicating that each ecotype does not constitute a single panmictic group.

ISOLATION-BY-ADAPTATION

Genetic distances estimated by pooling across AFLP loci did not exhibit significant IBA or IBD for any class of loci or index of adaptive divergence (Table 4). In contrast, mtDNA often exhibited both IBA and IBD. However, the locus-specific analyses revealed that a substantial proportion of individual AFLP loci were indeed strongly positively correlated with adaptive divergence, thereby exhibiting the pattern of IBA (results from raw matrices are in Table 5; results using log-transformed matrices were highly congruent and are presented in the online Supplementary Material). Our main interest here is the correlation between genetic and adaptive divergence, independent of geographic distance (i.e., IBA). Thus, in the text we detail only the results from such analyses (i.e., the effects of adaptive divergence in partial Mantel tests). However, Table 5 provides results for all raw analyses, including simple Mantel tests and those involving geographic distance. Six analyses examined IBA, that is, three indices of adaptive divergence, with raw and log distances for each. On average, about 9% of AFLP loci exhibited significant IBA (for raw and log distances, respectively: Host preference = 10%, 7%; Ecomorphology = 9%, 9%; % *Ceanothus* = 8%, 9%). A representative example of the analyses is depicted in Figure 5.

Several lines of evidence indicate that the number of loci found to exhibit IBA is not an artifact of multiple comparisons. First, the proportion of such loci (roughly 10%) is twice that expected under multiple comparisons, assuming a critical alpha of 0.05. Thus, minimally 5% of loci appear to be affected by adaptive divergence. If none of the correlations are spurious, 10% are affected. Second, across the six analyses there were at least twice as many significant positive correlations as negative ones in five of six cases, and significantly more positive associations than

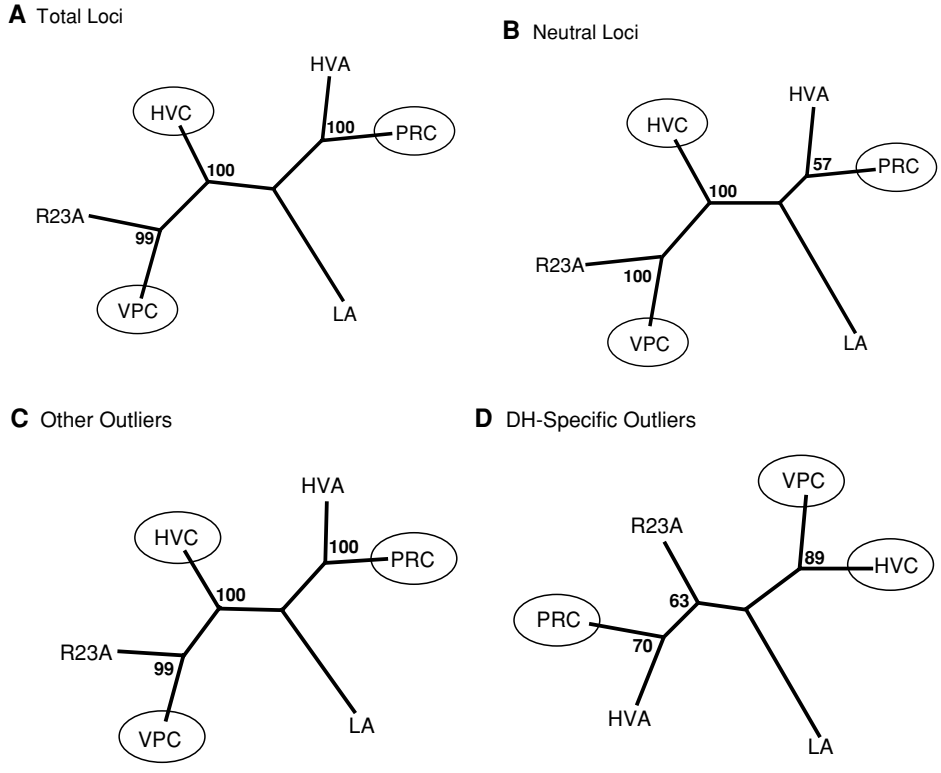

Figure 4. Unrooted neighbor-joining 50% majority rule consensus trees based on Nei's genetic distance between populations, calculated using 1000 bootstrap replicates in AFLP-SURV. Bootstrap values greater than 50% are indicated at nodes. The names of *Ceanothus* populations are circled for clarity. Results are depicted from four classes of loci: (A) Total Loci, (B) Neutral Loci, (C) Other Outliers, an (D) DH-Specific Outliers (see text for details of locus classes).

expected by chance in nine of 18 analyses (binomial tests, Table 5). Third, the distribution of correlation coefficients from significant loci had a positive mean in all cases, and a mean significantly greater than zero in five of six cases (with $P = 0.063$ for the sixth case).

Loci exhibiting IBA were not necessarily outlier loci. Across the six analyses, only 6–29% (mean = 16%) of the IBA associations were derived from outliers in the Dfdist analyses (Table 5). Thus, IBA was repeatedly detected for putatively neutral nonoutlier loci. Indeed, these loci generally exhibited F_{ST} values well below the 95% quantile. Considering only the 155 nonoutlier loci, about 10% exhibited significant IBA (for raw and log distances, respectively: Host Preference = 10%, 6%; Ecomorphology = 10%, 10%; % *Ceanothus* = 10%, 10%). Finally, we note that results from simple Mantel tests of genetic versus adaptive divergence were congruent with those from partial Mantel tests (Table 5).

Discussion

ECOLOGICAL SPECIATION AND COMPARATIVE GENOME SCANS

Past genome scans have valuably shown that divergence can be variable across the genome, with a few percent of loci exhibiting stronger differentiation than the remainder (2–10% of all loci were detected as outliers in at least one comparison in: Wilding et al. 2001; Emelianov et al. 2003; Campbell and Bernatchez 2004; Scotti-Saintagne et al. 2004; Acheré et al. 2005; Turner et al. 2005; Vasemagi et al. 2005; Bonin et al. 2006; Murray and Hare 2006; Savolainen et al. 2006; Yatabe et al. 2007; S. P. Egan, P. Nosil, and D. J. Funk, unpubl. ms.). Our genome scan detected various types of outliers (Table 3 for summary). Consistent with past work, we found that a relatively small proportion of loci (8%) were observed as outliers in multiple population comparisons. These "repeated outliers" are very likely subject to divergent selection, and unlikely to represent false positives (Campbell and Bernatchez 2004; Bonin et al. 2006). We also detected a number of "nonrepeated outliers" that appeared in only one comparison. These could reflect host-independent selection, but could also represent cases in which different genes/mutations are involved in host-associated adaptation of different populations. In this case, our estimate of the proportion of the genome tightly enough linked to selected regions to exhibit outlier behavior rises from 8% to 15%. However, note that only 0.7–5.1% (mean 3.8%) of loci appeared as outliers for any individual population pair (0.7–4.5%, mean = 2.6% when only different-host pairs are considered).

Our use of replicated ecologically divergent (different-host) and ecologically similar (same-host) population pairs allowed us

Table 4. Results of simple and partial Mantel tests examining how F_{ST}, estimated using all AFLP loci within a class, is related to indices of adaptive divergence and to geographic distance. Results are also shown for mtDNA divergence, where the only significantly positive associations were detected (in bold). The simple Mantel test of genetic distance against geographic distance is the same for each index of adaptive divergence. AD = Adaptive Divergence. GeoD = Geographic Distance. Analogous analyses using log-transformed distance matrices are presented in online Supplementary Material.

	Class of AFLP loci									
	Total Loci		Neutral Loci		Other Outlier		DH-Specific Outliers		mtDNA	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
GeoD simple										
All indices	0.30	0.31	0.20	0.32	0.45	0.09	−0.10	0.47	**0.73**	**0.03**
GeoD partial										
Host Preference	0.30	0.21	0.19	0.30	0.45	0.09	−0.09	0.39	0.59	0.08
Ecomorphology	0.30	0.31	0.20	0.33	0.45	0.11	−0.11	0.48	**0.81**	**0.02**
% Ceanothus	0.30	0.33	0.20	0.35	0.46	0.09	−0.11	0.47	**0.88**	**<0.01**
AD simple										
Host Preference	0.10	0.34	0.07	0.38	0.14	0.29	−0.05	0.47	**0.78**	**<0.01**
Ecomorphology	−0.05	0.49	−0.03	0.49	−0.13	0.39	0.20	0.21	0.43	0.06
% Ceanothus	−0.04	0.49	−0.01	0.53	−0.11	0.39	0.05	0.34	**0.59**	**<0.01**
AD partial										
Host Preference	−0.08	0.47	−0.05	0.49	−0.14	0.33	0.01	0.36	**0.66**	**0.01**
Ecomorphology	−0.05	0.49	−0.04	0.50	−0.15	0.37	0.20	0.21	**0.63**	**<0.01**
% Ceanothus	−0.06	0.48	−0.01	0.52	−0.15	0.36	0.05	0.34	**0.82**	**<0.01**

GeoD simple = simple Mantel of F_{ST} vs. geographic distance.
GeoD partial = partial Mantel of F_{ST} vs. geographic divergence, controlling for adaptive divergence.
AD simple = simple Mantel of F_{ST} vs. adaptive divergence.
AD partial = partial Mantel of F_{ST} vs. adaptive divergence, controlling for geographic distance.

to isolate the role of a specific ecological variable (host use) in causing outlier behavior, an approach not yet in widespread use. Most specifically, the DH-Specific Outliers detected (1% of total loci) are strong candidates for divergence via host-related selection (Funk 1998; Funk et al. 2002). A particularly strong candidate is the locus that was an outlier in two of three possible independent comparisons. In general, however, individual DH-Specific Outliers were not observed in a high proportion of different-host comparisons (Fig. 2). This moderate level of replication might reflect how host-associated populations of *T. cristinae* originated. If the use of each host arose only once, one might expect DH-Specific Outliers to be highly repeated among different-host pairs, because the single host shift involved divergence in specific mutations followed by their spread to multiple localities. In contrast, if associations with each host arose multiple times, then different mutations are more likely to have been involved in the divergence of populations in different geographic regions (Panova et al. 2006). Indeed, the selected loci (i.e., genes) might be the same under the multiple-origin scenario, but with divergence involving different mutations in these genes. In turn, these different mutations could either be recently derived or reflect the differential sorting of coexisting alleles from the ancestral gene pool. Evidence for the multiple origins scenario receives support from the lack of host-associated monophyly in phylogenies based on DNA sequences (Nosil et al. 2002) or AFLPs (this study, Fig. 4; P. Nosil, N. A. Spiegel, and D. J. Funk, unpubl. data from an expanded sampling of *T. cristinae* populations). The "mixed" outliers that appear in both different-host and same-host comparisons provide more of an enigma. These may be entirely responding to host-independent selection. Alternatively, they could have diverged under host-associated selection and then been transferred to same-host populations via gene flow.

Our analyses of linkage disequilibrium and phylogeographic structure resulted in some patterns that differed from past studies. Levels of pairwise disequilibria were generally low (≤ 0.1, Fig. 3) when compared, for example, to values of R in hybrid zone studies, which often range up to 0.6 even for unlinked loci (Szymura and Barton 1986; Mallet et al. 1990; Dasmahapatra et al. 2002). This suggests that the outlier loci detected in our study are not strongly clustered within the genome, for example on sex chromosomes or within chromosomal inversions. Nonetheless, our evidence for elevated linkage disequilibrium for Outlier Loci versus Neutral Loci in both allopatric and parapatric populations is consistent with some physical linkage among selected loci (Kim and Nielsen 2004). A pattern of elevated linkage disequilibrium for outlier loci relative to nonoutliers was reported for ecotypes of *Littorina saxatilis*, but only in the center of the contact zone in

Table 5. Summary of the distribution of Mantel correlation coefficients from analyses of F_{ST} at individual loci against adaptive divergence and geographic distance ($n = 209$ loci, of which 155 were nonoutliers in the genome scan). Binomial tests examine whether significant associations were positive more often than expected by chance. One-sample *t*-tests examine whether the mean of a distribution differs from zero (df = 1 minus the sample size). Significant results are in bold. The simple Mantel test of genetic distance against geographic distance is the same for each index of adaptive divergence. AD = Adaptive Divergence. GeoD = Geographic Distance. No. sig. + = number of loci exhibiting a significant positive relationships at $P < 0.05$; in parentheses are the number of loci with significant positive associations that were outliers in the Dfdist analyses. No. sig. − = number of loci exhibiting a significant negative relationship at $P < 0.05$. Analogous analyses using log-transformed distance matrices are presented in online Supplementary Material.

	No. sig. +	No. sig. −	Binomial *p*	Significant Loci Only			All Loci		
				Mean	*t*-test	*P*	Mean	*t*-test	*P*
GeoD simple									
All indices	19 (3)	10	0.136	0.33	3.40	**0.002**	0.13	4.23	**<0.001**
GeoD partial									
Host Preference	26 (6)	5	**<0.001**	0.53	6.15	**<0.001**	0.12	4.18	**<0.001**
Ecomorphology	19 (4)	8	0.052	0.39	3.88	**0.001**	0.13	4.24	**<0.001**
% Ceanothus	18 (3)	11	0.265	0.29	2.97	**0.006**	0.12	4.04	**<0.001**
AD simple									
Host Preference	18 (2)	7	**0.043**	0.37	3.62	**0.001**	0.07	3.31	**0.001**
Ecomorphology	12 (2)	6	0.238	0.24	2.28	**0.036**	0.01	0.88	0.378
% Ceanothus	9 (1)	7	0.804	0.13	1.24	0.233	0.03	1.77	0.078
AD partial									
Host Preference	20 (4)	7	**0.019**	0.30	3.26	**0.003**	0.01	0.68	0.499
Ecomorphology	18 (3)	6	**0.023**	0.27	3.03	**0.006**	0.01	0.20	0.841
% Ceanothus	17 (1)	11	0.345	0.17	1.94	0.063	0.02	0.90	0.368

GeoD simple = simple Mantel of F_{ST} vs. geographic distance.
GeoD partial = partial Mantel of F_{ST} vs. geographic divergence, controlling for adaptive divergence.
AD simple = simple Mantel of F_{ST} vs. adaptive divergence.
AD partial = partial Mantel of F_{ST} vs. adaptive divergence, controlling for geographic distance.

which migration between differentiated populations likely generates the observed linkage disequilibrium (Grahame et al. 2006). That study thus provided evidence for statistical, rather than physical, linkage.

With respect to phylogenetic structure, most past studies have found that putatively Neutral Loci group populations by geography, whereas Outlier Loci group populations in a manner consistent with the monophyly of ecologically similar populations (Wilding et al. 2001; Campbell and Bernatchez 2004; Dopman et al. 2005; Bonin et al. 2006; S. P. Egan, P. Nosil, and D. J. Funk, unpubl. ms.). In contrast, we found less evidence for phylogenetic grouping by host in analyses of selected loci. This result likely reflects the few (five) DH-Specific Outliers and their distribution across no more than four of nine different-host comparisons. Thus, this study presents a case in which the inclusion of selected loci is unlikely to confound phylogenetic analysis.

EXPLANATIONS FOR ISOLATION BY ADAPTATION

We found that up to 10% of nonoutlier AFLP loci were significantly and positively correlated with levels of adaptive divergence, thereby exhibiting IBA. Two potentially complementary biological explanations for these patterns will be considered here. The first of these is general barriers to neutral gene flow as a function of the reproductive isolation promoted by adaptive divergence. The second is the effects of selection on weakly physically linked neutral regions. Under both these hypotheses, divergent selection plays a role in the differentiation of functionally neutral loci that are not tightly linked to genes under selection.

The general barriers hypothesis assumes that gene flow occurs between populations, but that gene flow is nonetheless restricted by divergent adaptation and thus decreases with increasing adaptive divergence. In *T. cristinae*, gene flow is known to occur between adjacent, parapatric populations (Sandoval 1994a; Nosil et al. 2003; Nosil and Crespi 2000) and could occur on a larger spatial scale because reproductive isolation between populations is incomplete (Nosil 2007). Evidence for the general barriers hypothesis is provided by our demonstration of a geography-independent association between adaptive and mtDNA divergence. Because mtDNA is presumably neutral and is unlinked to the nuclear genome, this association seems likely to have arisen through the increasingly restricted gene flow that accompanies increasing adaptive divergence. However, if such a general adaptive barrier does exist, why did only 5–10% of AFLP loci exhibit IBA? Recall that barrier-induced divergence of neutral loci proceeds via

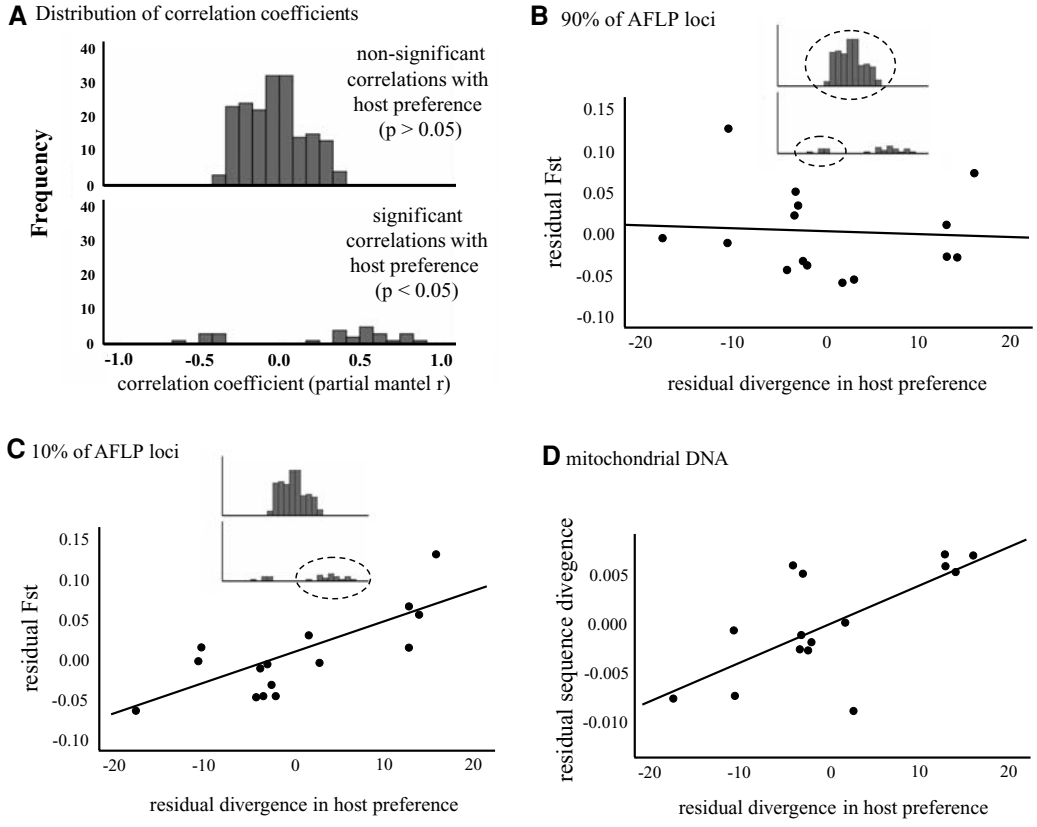

Figure 5. Representative results of tests for Isolation-By-Adaptation (see Tables 4 and 5 for full results). Shown are results using host preference as the index of adaptive divergence, but results were similar for other indices. Both axes in the scatter plots show residual values where correlations with geographic distance have been accounted for (i.e., representing partial plots). (A) A distribution of correlation coefficients from partial Mantel tests in which F_{ST} at each individual locus was examined against divergence in host preference, controlling for geographic distance. (B) The 90% of loci that did not exhibit significant IBA. The plot shows F_{ST} for these loci against divergence in host preference. (C) The 10% of loci that exhibited significant IBA. The plot shows mean F_{ST} for these loci against divergence in host preference. (D) Sequence divergence in mtDNA against divergence in host preference. See text for analytical details.

genetic drift. Thus, one explanation is that the barrier has arisen too recently for a higher proportion of neutral loci to have stochastically and substantially differentiated, even between the most adaptively divergent populations. However, this argument is weakened by nontrivial F_{ST} values (0.02–0.18 across loci, mean = 0.09) and levels of sequence divergence between allopatric populations (mean = 3–4%, 1–2% at COI and ITS-2, respectively; Nosil et al. 2002, 2003) consistent with up to two million years of divergence (based on the arthropod mtDNA clock of Brower 1994). Alternatively the barrier may be permeable and population sizes are too large to allow more general differentiation in the face of extant gene flow, episodes of historical gene flow may have eroded divergence at many neutral loci, or stabilizing selection constrains divergence.

One outstanding question concerning neutral gene flow in the wild is the degree to which it is reduced by geographic distance (resulting in IBD) versus adaptive divergence (resulting in IBA) (Crispo et al. 2006). We found that both factors may be equally important, with 10% of loci significantly exhibiting each pattern (Table 5). The distributions of correlation coefficients across all loci in the Mantel analyses were shifted toward being positive for analyses of geographic versus genetic distance, but not for adaptive versus genetic divergence. This pattern might reflect more loci being affected by geography than adaptive divergence. Other studies have also found that both geographic distance and adaptive divergence influence gene flow (Wilding et al. 2001; Grahame et al. 2006; Parchman et al. 2006; Pilot et al. 2006). However, some studies report a strong role for geographic distance alone (Smith et al. 2005; Crispo et al. 2006) or for adaptive divergence alone (Lu and Bernatchez 1999; Cooper 2000; Ogden and Thorpe 2002; Rocha et al. 2005). Studies aimed at determining the causes of this variability among systems would be of interest. We note, however, that geography and selection are complementary, rather than opposing, causes of divergence. Another topic of interest is causality—does adaptive divergence reduce gene flow (as discussed in the current study), or vice-versa? Adaptive divergence could be constrained by gene flow, similarly resulting in correlations between adaptive and genetic divergence (Hendry

and Taylor 2004; Nosil and Crespi 2004). However, the lack of an association between overall F_{ST} across all loci and levels of adaptive divergence argues against this interpretation in the current study. Moreover, our index of adaptive divergence in one of our assayed ecological traits, host use (based upon the composition of host plant patches), is not a phenotypic property of *T. cristinae*, and thus is unlikely to be constrained by gene flow between insect populations.

The other major mechanism by which genetic divergence at nonoutlier loci could become associated with adaptive divergence involves physical linkage. If neutral regions are physically linked on the chromosome to genes under selection, three outcomes might be observed in a genome scan. First, if they are tightly linked, they might differentiate enough to be detected as outliers themselves. Second, if they are more weakly linked, they might exhibit somewhat elevated differentiation relative to neutral expectations, but not so much as to be identified as outliers. In this case, their degree of differentiation should be associated with the degree of selection on the loci to which they are linked and on the physical proximity of their linkage. Third, if loci are very distantly linked to genes under selection, identifiably elevated differentiation may not occur. Here, we argue that the 10% of neutral loci exhibiting IBA could represent the second class, of weakly linked loci. Theory supports this possibility. Charlesworth et al. (1997) found that with population subdivision, local selection increases genetic differentiation even at neutral sites relatively distant from the locus under selection. Although they found that divergence was most pronounced at sites close to the selected locus, the effects extended along the chromosome and did not disappear with high recombination rates.

The roles of general barriers versus weak linkage can potentially be distinguished with information on the spatial scale of gene flow. If the distribution of our study populations exceeds the actual scale of gene flow, then genetic divergence must have occurred in its absence, entirely by selection and drift. In this case, loci showing associations with adaptive divergence are best interpreted as having evolved through linkage to genes under selection. One means of addressing this issue is by asking whether gene flow into allopatric populations occurs. If it does not, then the general barriers argument is weakened. The maintenance of maladaptive phenotypes within allopatric *T. cristinae* populations, despite strong selection against them, is consistent with gene flow into allopatry (Nosil 2004; Bolnick and Nosil, in press). The elevated linkage disequilibrium observed for outlier loci in allopatric populations could also be explained by migration into allopatric populations. Conversely, quantitative genetic data suggest low or no gene flow into allopatry. Migration between populations generates genetic covariance between phenotypic traits that differ between populations. A lack of genetic covariance between color pattern and host preference in allopatry, despite strong genetic covariance in parapatry, suggests no more than minimal gene flow into allopatry (Nosil et al. 2006b). In summary, certain observations favor each scenario. This need not be viewed as problematic as general barriers and weak linkage might act in conjunction. To evaluate their relative contributions, future studies could examine the spatial scale at which gene flow occurs. Studies employing microsatellites, and coalescent-based approaches that distinguish between divergence with versus without gene flow (e.g., Nielsen and Wakeley 2001; Hey and Nielsen 2004), could address the spatial scale of *T. cristinae* gene flow explicitly. Additionally, QTL mapping could determine the physical genomic distribution of loci exhibiting associations with adaptive divergence to evaluate arguments on linkage. No matter the explanation, our findings clearly show that up to 10% of the neutral genome has been affected by adaptive divergence.

A final point concerns loci exhibiting IBA that were also outliers in the genome scan. In all instances, these represented loci that appeared as outliers in both different-host and same-host pairs. Based on this distribution alone, these loci could have been subject to any combination of host-related and host-independent selection, and may not have been influence by host-related selection at all. However, our indices of adaptive divergence are based on aspects of host-specific ecology. Furthermore, these mixed outliers exhibited ecotype-associated differences in the Structure analyses. Thus, it is quite likely that outliers exhibiting IBA have specifically been differentiating under host-related selection, with alleles transferred between populations via gene flow. If so, our minimum estimate of loci subject to host-related selection that was based on the DH-Specific Outliers of (∼1%) can be revised to roughly 2% by including the outlier loci exhibiting IBA (Table 5).

Conclusions and Future Directions

Our comparative approach allowed us to alleviate concerns that have recently been raised about identifying loci under selection using genome scans (e.g., type I error, mutation rate variation). By assessing loci observed to be outliers in multiple comparisons and those restricted to different-host comparisons we were able to confidently identify outlier loci subject to natural selection (8%) and determine which of these were affected by host-related selection specifically (1–2%). Importantly, by taking a locus-specific approach and using available data on phenotypic traits under divergent selection, we were able to quantify the proportion of the neutral genome affected by adaptive divergence (5–10%). This study demonstrates how the combination of population-level data on both ecological phenotypes and multilocus genotypes can demonstrate the specific contributions of divergent host adaptation on genomic differentiation during ecological speciation. Herbivorous insects have long been used to study speciation through the analysis of reproductive barriers (Via 1999; Berlocher and Feder 2002;

Drès and Mallet 2002; Funk et al. 2002; Funk and Nosil 2007). Our results illustrate how genome scans can be applied to evaluate the genetics of host adaptation and ecological speciation in these organisms.

Our results further illustrate how genomic divergence during speciation can reflect the joint effects of fundamental evolutionary processes, that is selection, drift, and gene flow. Selection itself may have played multiple roles in our study populations by acting on functionally important genes and those tightly linked to them, by reducing gene flow and thereby facilitating divergence through drift, and by affecting the differentiation of neutral regions that are weakly linked to those under selection. Future work should proceed in two major directions. From a population genomics perspective it would be informative to determine the factors that control the proportion of the neutral genome affected by adaptive divergence, and to further evaluate the mechanisms by which correlations between adaptive and genetic divergence arise. From a molecular genetics perspective, our robust identification of candidate loci involved in divergent host adaptation and speciation paves the way for mapping and sequencing studies aimed at verifying the specific gene regions subject to selection, and identifying functionally important genetic variation (Nielsen 2005; Vasemagi and Primmer 2005; Noor and Feder 2006; Stinchcombe and Hoekstra 2007).

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LITERATURE CITED

Achere, V., J. M. Favre, G. Besnard, and S. Jeandroz. 2005. Genomic organization of molecular differentiation in Norway spruce (*Picea abies*). Mol. Ecol. 14:3191–3201.

Arnold, S. J. 1992. Constraints on phenotypic evolution. Am. Nat. 140:S85–S107.

Balloux, F., and N. Lugon-Moulin. 2002. The estimation of population differentiation with microsatellite markers. Mol. Ecol. 11:155–165.

Barton, N., and B. O. Bengtsson. 1986. The barrier to genetic exchange between hybridizing populations. Heredity 57:357–376

Beaumont, M. A. 2005. Adaptation and speciation: what can F_{ST} tell us? Trends Ecol. Evol. 20:435–440.

Beaumont, M. A., and D. J. Balding. 2004. Identifying adaptive genetic divergence among populations from genome scans. Mol. Ecol. 13:969–980.

Beaumont, M. A., and R. A. Nichols. 1996. Evaluating loci for use in the genetic analysis of population structure. Proc. R. Soc. Lond. B 263:1619–1626.

Berlocher, S. H., and J. L. Feder. 2002. Sympatric speciation in phytophagous insects: moving beyond controversy? Ann. Rev. Entomol. 47:773–815.

Black, W. C., C. F. Baer, M. F. Antolin, and N. M. DuTeau. 2001. Population genomics: genome-wide sampling of insect populations. Ann. Rev. Entomol. 46:441–469.

Bohonak, A. J. 2002. IBD (isolation by distance): a program for analyses of isolation by distance. J. Hered. 93:153–154.

Bolnick, D. A., and P. Nosil. 2007. Natural selection in populations subject to a migration load. Evolution 61:2229–2243.

Bonin, A., E. Bellemain, P. B. Eidesen, F. Pompanon, C. Brochmann, and P. Taberlet. 2004. How to track and assess genotyping errors in population genetics studies. Mol. Ecol. 13:3261–3273.

Bonin, A., P. Taberlet, C. Miaud, and F. Pompanon. 2006. Explorative genome scan to detect candidate loci for adaptation along a gradient of altitude in the common frog (*Rana temporaria*). Mol. Biol. Evol. 23:773–783.

Bonin, A., D. Ehrich, and S. Manel. 2007. Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionist. Mol. Ecol. 16:3737–3758.

Bowcock, A. M., J. R. Kidd, J. L. Mountain, J. M. Hebert, L. Carotenuto, K. K. Kidd, and L. L. Cavalli-Sforza. 1991. Drift, admixture and selection in human evolution—a study with DNA polymorphisms. Proc. Natl. Acad. Sci. USA 88:839–843.

Bradshaw, H. D., and D. W. Schemske. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. Nature 426:176–178.

Brower, A. V. Z. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius-erato* inferred from patterns of mitochondrial-DNA evolution. Proc. Natl. Acad. Sci. USA 91:6491–6495.

Campbell, D., and L. Bernatchez. 2004. Generic scan using AFLP markers as a means to assess the role of directional selection in the divergence of sympatric whitefish ecotypes. Mol. Biol. Evol. 21:945–956.

Castellano, S., and E. Balletto. 2002. Is the partial Mantel test inadequate? Evolution 56:1871–1873.

Charlesworth, B., M. Nordborg, and D. Charlesworth. 1997. The effects of local selection, balanced polymorphism and background selection on equilibrium patterns of genetic diversity in subdivided populations. Genet. Res. 70:155–174.

Colosimo, P. F., K. E. Hosemann, S. Balabhadra, G. Villarreal, M. Dickson, J. Grimwood, J. Schmutz, R. M. Myers, D. Schluter, and D. M. Kingsley. 2005. Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. Science 307:1928–1933.

Cooper, M. L. 2000. Random amplified polymorphic DNA analysis of southern brown bandicoot (*Isoodon obesulus*) populations in Western Australia reveals genetic differentiation related to environmental variables. Mol. Ecol. 9:469–479.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Inc. Sunderland, MA.

Crespi, B. J., and C. P. Sandoval. 2000. Phylogenetic evidence for the evolution of ecological specialization in *Timema* walking-sticks. J. Evol. Biol. 13:249–262.

Crispo, E., P. Bentzen, D. N. Reznick, M. T. Kinnison, and A. P. Hendry. 2006. The relative influence of natural selection and geography on gene flow in guppies. Mol. Ecol. 15:49–62.

Dasmahapatra, K. K., M. J. Blum, A. Aiello, S. Hackwell, N. Davies, E. P. Bermingham, and J. Mallet. 2002. Inferences from a rapidly moving hybrid zone. Evolution 56:741–753.

Dettman, J. R., C. Sirjusingh, L. M. Kohn, and J. B. Anderson. 2007. Incipient speciation by divergent adaptation and antagonistic epistasis in yeast. Nature 447:585–588.

Dobzhansky, T. 1951. Genetics and the origin of species. 3rd edn. Columbia Univ. Press, New York.

Dopman, E. B., L. Perez, S. M. Bogdanowicz, and R. G. Harrison. 2005. Consequences of reproductive barriers for genealogical discordance in the European corn borer. Proc. Natl. Acad. Sci. USA 102:14706–14711.

Drès, M., and J. Mallet. 2002. Host races in plant-feeding insects and their importance in sympatric speciation. Phil. Trans. R. Soc. Lond. B: 357:471–492.

Emelianov, I., F. Marec, and J. Mallet. 2003. Genomic evidence for divergence with gene flow in host races of the larch budmoth. Proc. R. Soc. Lond. B 271:97–105.

Falush, D., M. Stephens, and J. K. Pritchard. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol. Ecol. Notes 7:574–578.

Feder, J. L., S. B. Opp, B. Wlazlo, K. Reynolds, W. Go, and S. Spsisak. 1994. Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. Proc. Natl. Acad. Sci. USA 91:7990–7994.

Feder, J. L., F. B. Roethele, K. Filchak, J. Niedbalski, and J. Romero-Severson. 2003. Evidence for inversion polymorphism related to sympatric host race formation in the apple maggot fly, *Rhagoletis pomonella*. Genetics 163:939–953.

Felsenstein, J. 2004. PHYLIP. Distributed by the author, Department of Genome Sciences, Univ. of Washington, Seattle, WA.

Funk, D. J. 1998. Isolating a role for natural selection in speciation: host adaptation and sexual isolation in *Neochlamisus bebbianae* leaf beetles. Evolution 52:1744–1759.

Funk, D. J., and P. Nosil. 2007. Comparative analyses and ecological speciation in herbivorous insects. Pp. 117–135 *in* K. Tilmon, ed. Ecology and evolution of herbivorous insects California Univ. Press, CA.

Funk, D. J., K. E. Filchak, and J. L. Feder. 2002. Herbivorous insects: model systems for the comparative study of speciation ecology. Genetica 116:251–267.

Funk, D. J., P. Nosil, and W. Etges. 2006. Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. Proc. Natl. Acad. Sci. USA 103:3209–3213.

Gavrilets, S. 2004. Fitness landscapes and the origin of species. Princeton Univ. Press, Princeton, NJ.

Gavrilets, S., and M. B. Cruzan. 1998. Neutral gene flow across single locus clines. Evolution 52:1277–1284.

Gavrilets, S., and A. Vose. 2005. Dynamic patterns of adaptive radiation. Proc. Natl. Acad. Sci. USA 102:18040–18045.

Grahame, J. W., C. S. Wilding, and R. K. Butlin. 2006. Adaptation to a steep environmental gradient and an associated barrier to gene exchange in *Littorina saxatilis*. Evolution 60:268–278.

Hartl, D. L., and A. G. Clark. 1997. Principles of population genetics, 3rd edn. Sinauer Associates, Inc. Sunderland, MA.

Haubold, B., and R. R. Hudson. 2000. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Bioinformatics 16:847–848.

Hawthorne, D. J., and S. Via. 2001. Genetic linkage of ecological specialization and reproductive isolation in pea aphids. Nature 412:904–907.

Hedrick, P. W. 2005. A standardized genetic differentiation measure. Evolution 59:1633–1638.

———. 2006. Genetic polymorphism in heterogeneous environments: The age of genomics. Annu. Rev. Ecol. Syst. 37:67–93.

Hendry, A. P., and E. B. Taylor. 2004. How much of the variation in adaptive divergence can be explained by gene flow? An evaluation using lake-stream stickleback pairs. Evolution 58:2319–2331.

Hey, J., and R. Nielsen. 2004. Multilocus methods for estimating population sizes, migration rates and divergence time, with applications to the divergence of *Drosophila pseudoobscura* and *D-persimilis*. Genetics 167:747–760.

Hill, W. G. 1974. Estimation of linkage disequilibrium in randomly mating populations. Heredity 33:229–239.

Hoekstra, H. E., R. J. Hirschmann, R. A. Bundey, P. A. Insel, and J. P. Crossland. 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. Science 313:101–104.

Jiggins, C. D., R. E. Naisbit, R. L. Coe, and J. M. Mallet. 2001. Reproductive isolation caused by colour pattern mimicry. Nature 411:302–305.

Joron, M., R. Papa, M. Beltran, N. Chamberlain, J. Mavarez, S. Baxter, M. Abanto, E. Bermingham , S. J. Humphray, J. Rogers, et al. 2006. A conserved supergene locus controls colour pattern diversity in *Heliconius* butterflies. PLOS Biol. 4:1831–1840.

Kim, Y., and R. Nielsen. 2004. Linkage disequilibrium as a signature of selective sweeps. Genetics 167:1513–1524.

Kimura, M. 1956. A model of genetic system which leads to closer linkage by natural selection. Evolution 10:278–287.

———. 1980. A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111–120.

Kirkpatrick, M., T. Johnson, and N. Barton. 2002. General models of multilocus evolution. Genetics 161:1727–1750.

Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5:150–163.

Lu, G. Q., and L. Bernatchez. 1999. Correlated trophic specialization and genetic divergence in sympatric lake whitefish ecotypes (*Coregonus clupeaformis*): support for the ecological speciation hypothesis. Evolution 53:1491–1505.

Lynch, M., and B. G. Milligan. 1994. Analysis of population genetic structure with RAPD markers. Mol. Ecol. 3:91–99.

Luikart, G., P. R. England, D. Tallmon, S. Jordan, and P. Taberlet. 2003. The power and promise of population genomics: from genotyping to genome typing. Nat. Rev. Genet. 4:981–994.

MacCallum, C. J., B. Nürnberger, N. H. Barton, and J. M. Szymura. 1998. Habitat preference in a *Bombina* hybrid zone in Croatia. Evolution 52:227–239.

Mallet, J., and N. H. Barton. 1989. Strong natural selection in a warning-color hybrid zone. Evolution 43:421–431.

Mallet, J., N. Barton, G. L. M., C. Jose Santisteban, M. Manuel Muedas, and H. Eeley. 1990. Estimates of selection and gene flow from measures of cline width and linkage disequilibrium in *Heliconius* hybrid zones. Genetics 124:921–936.

Manly, B. F. J. 1997. Randomization and Monte Carlo methods in biology. 2nd edn. Chapman and Hall, New York.

Mayr, E. 1947. Ecological factors in speciation. Evolution 1:263–288.

———. 1963. Animal Species and Evolution. Harvard Univ. Press, Cambridge.

Muller, H. J. 1940. Bearings of the *Drosophila* work on systematics. Pp. 185–268 *In* J. S. Huxley, ed. The new systematics. Clarendon Press, Oxford.

———. 1942. Isolating mechanisms, evolution and temperature. Biol. Symp. 6:71–125.

Murray, M. C., and M. P. Hare. 2006. A genomic scan for divergent selection in a secondary contact zone between Atlantic and Gulf of Mexico oysters, *Crassostrea virginica*. Mol. Ecol. 15:4229–4242.

Nachman, M. W., H. E. Hoekstra, and S. L. D'Agostino. 2003. The genetic basis of adaptive melanism in pocket mice. Proc. Natl. Acad. Sci. USA 100:5268–5273.

Nei, N. and W-H Li. 1973. Linkage disequilibrium in subdivided populations. Genetics 75:213–219.

Nielsen, R. 2005. Molecular signatures of natural selection. Ann. Rev. Genetics 39:197–218.

Nielsen, R., and J. Wakeley. 2001. Distinguishing migration from isolation: a Markov chain Monte Carlo approach. Genetics 158:885–896.

Noor, M. A. F., and J. L. Feder. 2006. Speciation genetics: evolving approaches. Nat. Rev. Genet. 7:851–861.

Noor, M. A. F., K. L. Grams, L. A. Bertucci, and J. Reiland. 2001. Chromosomal inversions and the reproductive isolation of species. Proc. Natl. Acad. Sci. USA 98:12084–12088.

Nosil, P. 2004. Reproductive isolation caused by visual predation on migrants between divergent environments. Proc. R. Soc. Lond. B 271:1521–1528.

———. 2007. Divergent host-plant adaptation and reproductive isolation between ecotypes of *Timema cristinae* walking-sticks. Am. Nat. 169:151–162.

Nosil, P., and B. J. Crespi. 2004. Does gene flow constrain trait divergence or vice-versa? A test using ecomorphology and sexual isolation in *Timema cristinae* walking-sticks. Evolution 58:101–112.

———. 2006a. Experimental evidence that predation promotes divergence during adaptive radiation. Proc. Natl. Acad. Sci. USA 103:9090–9095.

———. 2006b. Ecological divergence promotes the evolution of cryptic reproductive isolation. Proc. R. Soc. Lond. B 273:991–997.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. Nature 417:440–443.

———. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proc. R. Soc. Lond. B 270:1911–1918.

Nosil, P., T. H. Vines, and D. J. Funk. 2005. Perspective : reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution 59:705–719.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2006a. The evolution of host preferences in allopatric versus parapatric populations of *Timema cristinae*. J. Evol. Biol. 19:929–942.

Nosil, P., B. J. Crespi, C. P. Sandoval, and M. Kirkpatrick. 2006b. Migration and the genetic covariance between habitat preference and performance. Am. Nat. 167: E66–E78.

Nosil, P., B. J. Crespi, R. Gries, and G. Gries. 2007. Natural selection and divergence in mate preference during speciation. Genetica 129:309–327.

Ogden, R., and R. S. Thorpe. 2002. Molecular evidence for ecological speciation in tropical habitats. Proc. Natl. Acad. Sci. USA 99:13612–13615.

Orr, H. A. 1995. The population genetics of speciation: the evolution of hybrid incompatibilities. Genetics 139:1805–1813.

Panova, M., J. Hollander, and K. Johannesson. 2006. Site-specific genetic divergence in parallel hybrid zones suggests nonallopatric evolution of reproductive barriers. Mol. Ecol. 15:4021–4031.

Parchman, T. L., C. W. Benkman, and S. C. Britch. 2006. Patterns of genetic variation in the adaptive radiation of New World crossbills (Aves : Loxia). Mol. Ecol. 15:1873–1887.

Pialek, J., and N. H. Barton. 1997. The spread of an advantageous allele across a barrier: the effects of random drift and selection against heterozygotes. Genetics 145:493–504.

Pilot, M., W. Jedrzejewski, W. Branicki, V. E. Sidorovich, B. Jedrzejewska, K. Stachura, and S. M. Funk. 2006. Ecological factors influence population genetic structure of European grey wolves. Mol. Ecol. 15:4533–4553.

Pompanon, F., A. Bonin, E. Bellemain, and P. Taberlet. 2005. Genotyping errors: causes, consequences and solutions. Nat. Rev. Genet. 6:847–859.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Raufaste, N., and F. Rousset. 2001. Are partial mantel tests adequate? Evolution 55:1703–1705.

Rieseberg, L. H. 2001. Chromosomal rearrangements and speciation. Trends Ecol. Evol. 16:351–358.

Rocha, L. A., D. R. Robertson, J. Roman, and B. W. Bowen. 2005. Ecological speciation in tropical reef fishes. Proc. R. Soc. Lond. B 272:573–579.

Rousset, F. 2002. Partial Mantel tests: reply to Castellano and Balletto. Evolution 56:1874–1875.

Rundle, H., and P. Nosil. 2005. Ecological speciation. Ecol. Lett. 8:336–352.

Rundle, H. D., L. Nagel, J. W. Boughman, and D. Schluter. 2000. Natural selection and parallel speciation in sympatric sticklebacks. Science 287:306–308.

Rundle, H. D., S. M. Vamosi, and D. Schluter. 2003. Experimental test of predation's effect on divergent selection during character displacement in sticklebacks. Proc. Natl. Acad. Sci. USA 100:14943–14948.

Sandoval, C. P. 1994a. The effects of relative geographic scales of gene flow and selection on morph frequencies in the walking stick *Timema cristinae*. Evolution 48:1866–1879.

———. 1994b. Differential visual predation on morphs of *Timema cristinae* (Phasmatodeae: Timemidae) and its consequences for host range. Biol. J. Linn. Soc. 52:341–356.

———. 2000. Presence of a walking-stick population (Phasmatoptera: Timematodea) after a wildfire. Southwest. Nat. 45:19–25.

Sandoval, C. P., and P. Nosil. 2005. Counteracting selective regimes and host preference evolution in ecotypes of two species of walking-sticks. Evolution 59:2405–2413.

Savolainen, V., M. C. Anstett, C. Lexer, I. Hutton, J. J. Clarkson, M. V. Norup, M. P. Powell, D. Springate, N. Salamin, and W. J. Baker. 2006. Sympatric speciation in palms on an oceanic island. Nature 441:210–213.

Schlötterer, C. 2002. A microsatellite-based multilocus screen for the identification of local selective sweeps. Genetics 160:753–763.

Schluter, D. 1994. Experimental evidence that competition promotes divergence in adaptive radiation. Science 266:798–800.

———. 2000. The ecology of adaptive radiation. Oxford Univ. Press, Oxford.

Schluter, D., and L. M. Nagel. 1995. Parallel speciation by natural selection. Am. Nat. 146:292–301.

Scotti-Saintagne, C., S. Mariette, I. Porth, P. G. Goicoechea, T. Barreneche, K. Bodenes, K. Burg, and A. Kremer. 2004. Genome scanning for interspecific differentiation between two closely related oak species [*Quercus robur* L. and *Q. petraea* (Matt.) Liebl.]. Genetics 168: 1615–1626.

Smith, T. B., R. Calsbeek, R. K. Wayne, K. H. Holder, D. Pires, and C. Bardeleben. 2005. Testing alternative mechanisms of evolutionary divergence in an African rain forest passerine bird. J. Evol. Biol. 18:257–268.

Stinchcombe, J. R., and H. E. Hoekstra. 2007. Combining population genomics and quantitative genetics: finding the genes underlying ecologically important traits. Heredity. *In press*.

Szymura, J. M., and N. H. Barton. 1986. Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina bombina* and Bombina *variegata*, near Cracow in Southern Poland. Evolution 40:1141–1159.

Turner, T. L., M. W. Hahn, and S. V. Nuzhdin. 2005. Genomic islands of speciation in *Anopheles gambiae*. PLoS Biol. 3:1572–1578.

Vamosi, S. M. 2005. On the role of enemies in divergence and diversification of prey: a review and synthesis. Can. J. Zool. 83:894–910.

Vasemagi, A., and C. R. Primmer. 2005. Challenges for identifying functionally important genetic variation: the promise of combining complementary research strategies. Mol. Ecol. 14:3623–3642.

Vasemagi, A., J. Nilsson, and C. R. Primmer. 2005. Expressed sequence tag-linked microsatellites as a source of gene-associated polymorphisms for detecting signatures of divergent selection in Atlantic salmon (*Salmo salar* L.). Mol. Biol. Evol. 22:1067–1076.

Vekemans, X., T. Beauwens, M. Lemaire, and I. Roldan-Ruiz. 2002. Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. Mol. Ecol. 11:139–151.

Via, S. 1999. Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. Evolution 53:1446–1457.

———. 2001. Sympatric speciation in animals: the ugly duckling grows up. Trends Ecol. Evol. 16:381–390.

Vickery, V. R. 1993. Revision of *Timema* Scudder (Phasmatoptera: Timematodea) including three new species. Can. Entomol. 125:657–692.

Vines, T. H., S. C. Köhler, M. Thiel, I. Ghira, T. R. Sands, C. J. MacCallum, N. H. Barton, and B. Nürnberger. 2003. The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*. Evolution 57:1876–1888.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Vandelee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP—a new technique for DNA-fingerprinting. Nucl. Acids Res. 23:4407–4414.

Wilding, C. S., R. K. Butlin, and J. Grahame. 2001. Differential gene exchange between parapatric morphs of *Littorina saxatilis* detected using AFLP markers. J. Evol. Biol. 14:611–619.

Wright, S. 1931. Evolution in Mendelian populations. Genetics 16:97–159.

———. 1943. Isolation by distance. Genetics 28:114–138.

Wu, C. I. 2001. The genetic view of the process of speciation. J. Evol. Biol. 14:851–865.

Yatabe, Y., N. C. Kane, C. Scotti-Saintagne, and L. H. Rieseberg. 2007. Rampant gene exchange across a strong reproductive barrier between the annual sunflowers, *Helianthus annuus* and *H. petiolaris*. Genetics 175:1883–1893.

Zhivotovsky, L. A. 1999. Estimating population structure in diploids with multilocus dominant DNA markers. Mol. Ecol. 8:907–913.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Primer pairs used to generate the 534 repeatable, polymorphic AFLP loci used in our analyses.

Table S2. Baseline information about population variability.

Table S3. Detailed summary of behavior for all 78 loci detected as outliers at the 95% quantile level.

Table S4. Results of simple and partial mantel tests examining how F^{st}, estimated using all AFLP loci within a class, is related to indices of adaptive divergence and to geographic distance.

Table S5. Summary of the distribution of mantel correlation coefficients from analyses of FST at individual loci against adaptive divergence and geographic distance (n = 206, of which 155 were nonoutliers in the genome scan).

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