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GEOGRAPHIC VARIATION IN ADAPTATION AT THE MOLECULAR LEVEL: A CASE STUDY OF PLANT IMMUNITY GENES

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Natural selection imposed by interacting species frequently varies among geographic locations and can lead to local adaptation, where alternative phenotypes are found in different populations. Little is known, however, about whether geographically variable selection acting on traits that mediate species interactions is consistent or strong enough to influence patterns of nucleotide variation at individual loci. To investigate this question, we examined patterns of nucleotide diversity and population structure at 16 plant innate immunity genes, with putative functions in defending plants against pathogens or herbivores, from six populations of teosinte (*Zea mays* ssp. *parviglumis*). Specifically, we tested whether patterns of population structure and within-population diversity at immunity genes differed from patterns found at nonimmunity (reference) loci and from neutral expectations derived from coalescent simulations of structured populations. For the majority of genes, we detected no strong evidence of geographically variable selection. However, in the wound-induced serine protease inhibitor (*wip1*), which inhibits the hydrolysis of dietary proteins in insect herbivores, one population showed unusually high levels of genetic differentiation, very low levels of nucleotide polymorphism, and was fixed for a novel replacement substitution in the active site of the protein. Taken together, these data suggest that *wip1* experienced a recent selective sweep in one geographic region; this pattern may reflect local adaptation or an ongoing species-wide sweep. Overall, our results indicate that a signature of local adaptation at the molecular level may be uncommon—particularly for traits that are under complex genetic control.

KEY WORDS: **Host–parasite interactions, local adaptation, natural selection, nucleotide variation, population structure.**

Natural selection is a major force driving population divergence in ecologically important traits (e.g., Endler 1977; Linhart and Grant 1996; Mousseau et al. 2000; Reznick and Ghalambor 2001). Recently, the importance of spatially variable selection and local adaptation has received considerable attention in studies of species interactions and coevolution. This interest has been motivated in part by the recognition that species interactions often vary across landscapes (e.g., Kaltz and Shykoff 1998; Kraaijiveld and Godfray 1999; Thompson and Cunningham 2002; Zangerl and Berenbaum 2003; Rudgers and Strauss 2004; Heath and Tiffin 2007) and that traits that mediate species interactions are often differentiated among populations in parallel with variation in species interactions (e.g., Carrol and Boyd 1992; Brodie et al. 2002; Moeller 2006). Differences among populations in selection imposed by interacting species may reflect geographic variation in the abiotic and community context in which those interactions occur. Evidence of spatially variable selection and local adaptation of traits involved in species interactions have been documented at the phenotypic level (reviewed in Hoeksema and Forde 2008), but there have been few attempts to determine whether the genes

underlying these traits harbor evidence of geographically variable selection.

At the molecular level, local adaptation occurs when alternative alleles (or multilocus genotypes) are favored in different geographic locations, a process that would be expected to lead to low levels of nucleotide and allelic variation within populations under directional selection (throughout this article we refer to geographically distinct groups of intermating individuals as "populations" although these are often referred to as "subpopulations" in the population genetics literature). By contrast, when considering the broadest spatial scales (e.g., the species' range), geographical variation in selection and local adaptation has been considered a form of balancing selection that maintains alternative alleles within a species (Felsenstein 1976; Hedrick et al. 1976; Hedrick 1986). Therefore, geographically variable selection may lead to unusually low or high levels of nucleotide diversity depending on whether one examines diversity within a population or combines samples from multiple populations inhabiting contrasting selective environments. Geographically variable selection can also cause significantly higher levels of population genetic differentiation (e.g., F_{ST}) in focal (candidate) genes relative to other loci in the genome (Lewontin and Krakauer 1973; Taylor et al. 1995; Storz 2005; Stinchcombe and Hoekstra 2008)—a pattern that can be tested only if diversity is sampled from multiple populations. In principle, a signature of local adaptation at the molecular level could be detectable from sequence data; however, recent theoretical work has indicated that nucleotide polymorphism may often be unaffected by geographically variable selection or may be difficult to detect with standard tests of the neutral model of molecular evolution, particularly when phenotypes are under complex genetic control (Kelly 2006).

In this article, we use a survey of nucleotide variation in 16 plant immunity genes from six natural populations of the annual plant, teosinte (*Zea mays* ssp. *parviglumis*), to test for evidence of geographic variation in the history of natural selection on genes that play a role in defending plants against their natural enemies. The plant innate immune system is comprised of a recognition phase, which involves proteins that detect enemies, and a response phase, which involves proteins or secondary compounds that inhibit attack by altering plant physiology or directly interacting with enemies or their targets in the host (Dangl and Jones 2001; Jones and Dangl 2006). Sequence variation in genes involved in the plant immune system has been well characterized in several model systems (e.g., *Arabidopsis thaliana*, maize, tomato) and has been increasingly studied in the wild relatives of these model systems to test hypotheses about the process of coevolution at the molecular level (reviewed in deMeaux and Mitchell-Olds 2003; Tiffin and Moeller 2006). The majority of these studies have analyzed species-wide samples, and have revealed a variety of evolutionary histories. Some genes, predominantly R-genes involved in pathogen detection, show the signature of long-term balanced polymorphism (e.g., Stahl et al. 1999; Tian et al. 2002; Mauricio et al. 2003; Kroymann et al. 2003). Other immunity genes show evidence of species-wide selective sweeps (Bishop et al. 2000; Tiffin 2004; Tiffin et al. 2004), a pattern inconsistent with local adaptation. The majority of genes involved in immune response, however, harbor more complex patterns of sequence variation that may reflect spatial and temporal heterogeneity in the selective environment (Rose et al. 2004; Moeller and Tiffin 2005). In virtually all of these studies, the potential for geographically variable selection has been overlooked, or has been impossible to conclusively infer, because sequences were sampled from across species' ranges. Two exceptions, which have used surveys of molecular diversity to look for evidence of local adaptation in plant R genes, have not found strong evidence (de Meaux et al. 2003; Bakker et al. 2006).

The majority of the 16 genes we treat as immunity genes have been previously identified to be upregulated in maize in response to leaf wounding or pathogen infection and/or have been shown to function directly in resistance to pathogens or herbivores. These genes encode proteins with diverse biochemical function including chitinases, protease inhibitors, and ribosome-inactivating proteins (see Table 1 for putative functions and references; also Moeller and Tiffin 2005). Fifteen of these genes code for proteins that are classified as pathogenesis-related proteins; their expression is induced following infection by pathogens or damage by herbivores (Datta and Muthukrishnan 1999). Some of the defense proteins that we studied function as enzymes to confer resistance. For example, *mir1* is a cysteine protease produced by some maize lines that confers resistance to a variety of Lepidoptera by damaging the peritrophic matrix of caterpillar guts (Pechan et al. 2002). Other proteins function as enzyme inhibitors. For example, *mpi* codes for a protease inhibitor in maize that disrupts the function of digestive enzymes including chymotrypsin in caterpillars (*Spodoptera littoralis*) and elastase in mammals (Tamayo et al. 2000). Other proteins in our survey are expressed in response to fungal pathogen infection. PRMs are localized to the plasmodesmata of fungal-infected maize radicles (Murillo et al. 1997) and chitinases are hydrolytic enzymes that degrade chitin or glucan, major constituents of fungal cell walls (e.g., Schlumbaum et al. 1986). Some of these genes may also have roles in pathogen detection (e.g., chitinases). Previous analyses of these genes revealed unusually high levels of replacement polymorphism relative to silent polymorphism in range-wide samples, a pattern that may result from selection favoring different amino acids in different geographic locations, i.e. local adaptation (Moeller and Tiffin 2005).

In this study, we examined sequence diversity within multiple geographically distinct populations to explicitly test for evidence of local adaptation of plant immunity genes. Geographical

Table 1. Summary statistics for range-wide samples of immunity and reference loci including the size of the sequenced fragment, number of sequences (*N*), number of polymorphic sites (*S*), haplotype diversity (H_d), nucleotide diversity (π), the ratio of replacement to silent polymorphism (π_{rep}/π_{sil}), Tajima's *D*, population genetic differentiation (F_{ST}), and the population recombination rate (Hudson's (1987) 4Nr scaled by π) across all populations, and the protein class and/or function if known.

Locus	Fragment size	*N*	*S*	H_d	π	π_{rep}/π_{sil}	*D*	F_{ST}	4Nr/π	Protein Class/ Putative function	GenBank accession #s
Immunity											
chiA	1484	81	109	0.993	0.0106	0.373	−1.75	0.142	12.1	chitinase	EU724136–724216
chiB	1273	73	74	0.989	0.0130	0.311	−0.55	0.188	4.9	chitinase	EU274217–724289
chiI	1145	76	46	0.988	0.0054	0.088	−1.29	0.155	48.1	chitinase	EU724432–724507
hag	784	75	55	0.958	0.0094	0.441	−1.50	0.270	3.0	thaumatin-like protein	EU724290–724364
hm2	2536	76	63	0.889	0.0086	0.367	−0.80	0.046	0.6	HC-toxin degradation	EU724735–724809
mir1	1216	84	71	0.980	0.0123	0.452	−1.06	0.145	4.1	cysteine protease	EU724578–724661
mpi	699	70	25	0.948	0.0068	0.516	−0.83	0.196	17.5	protease inhibitor	EU724508–724577
plt2	672	73	37	0.963	0.0069	0.444	−1.52	0.120	12.3	lipid transferase	EU724662–724734
pr1	492	81	22	0.971	0.0082	0.111	−0.35	0.183	63.2	function unknown	EU724810–724890
pr5	616	84	27	0.963	0.0102	1.018	−0.26	0.194	4.3	thaumatin-like protein	EU725050–725133
pr6	1510	77	41	0.991	0.0068	0.308	−0.44	0.132	22.8	β-1,3 glucanase	EU724973–725049
prms	673	83	38	0.796	0.0091	0.376	−0.68	0.157	0.0	pr1 class	EU724891–724972
rip1	1373	76	103	0.973	0.0108	0.436	−1.49	0.207	5.7	ribosome-inactivating	EU725134–725209
rip2	681	75	41	0.988	0.0091	0.394	−1.12	0.255	62.3	ribosome-inactivating	EU725210–725284
wip1	1382	87	45	0.956	0.0157	0.208	−0.56	0.316	15.7	protease inhibitor	EU725370–725455
zlp	763	85	27	0.942	0.0038	0.139	−1.54	0.183	1150.3	thaumatin-like protein	EU725285–725369
Reference											
adh1	1530	73	112	0.987	0.0173	0.035	−0.21	0.170	3.6	alcohol dehydrogenase	EF539343–539415
asg65	949	78	68	0.972	0.0097	N/A	−1.11	0.102	0.9	noncoding	EF539416–539493
bnl7	975	71	56	0.949	0.0094	N/A	−0.83	0.414	0.2	noncoding	EF539494–539564
bz2	685	76	22	0.896	0.0076	0.309	0.21	0.170	0.3	anthocyanin biosynthesis	EU724060–724135
fus6	778	67	21	0.924	0.0097	0.060	−0.40	0.239	17.9	RFLP marker	EU724365–724431
glb	1266	81	184	0.991	0.0214	0.322	−1.57	0.124	12.0	embryo storage protein	EF539565–539645
waxy	1479	80	68	0.978	0.0091	0.054	−1.20	0.349	0.7	starch biosynthesis	EF539646–539725
Totals	24961	1796	1355		–	–		–		–	

variation in selection and local adaptation may be particularly relevant in *parviglumis* because populations (1) occur across a topographically variable region of southwest Mexico with strong environmental gradients and (2) exhibit moderate-to-high levels of neutral genetic differentiation and limited migration in some regions (Moeller et al. 2007). If geographically variable selection is more important in shaping diversity at the nucleotide level at immunity than nonimmunity genes, we expect immunity loci to have lower within-population diversity and higher population genetic structure than nonimmunity (reference) loci. We used DNA sequence data from the 16 immunity genes along with seven reference loci, which have no function in plant immunity, to test whether (1) patterns of nucleotide diversity and population genetic differentiation differ between the two classes of loci: immunity vs. reference loci, (2) population genetic parameters for individual immunity loci differ significantly from distributions derived from coalescent simulations of structured populations, and (3) population genetic parameters for individual immunity loci differ significantly from empirical distributions of population genetic parameters observed in reference loci.

Materials and Methods

SAMPLING OF POPULATIONS AND LOCI

Zea mays ssp. *parviglumis* Iltis and Doebley is a highly outcrossing annual plant endemic to southwest Mexico. We examined sequence variation within and among six natural populations. Four of these populations are in the western portion of its range (state of Jalisco, Mexico) and two are in central portion of its range in the Balsas River valley (state of Mexico, Mexico) (Fig. 1). These two regions are separated by ~300 km of mountainous terrain and the populations representing the two regions correspond to the two races of *parviglumis* distinguished by Wilkes (1967) based on morphometric traits. Populations within each region are also geographically separated and occur across a wide range of elevations and environments. Seeds were collected from 8 to 18 maternal

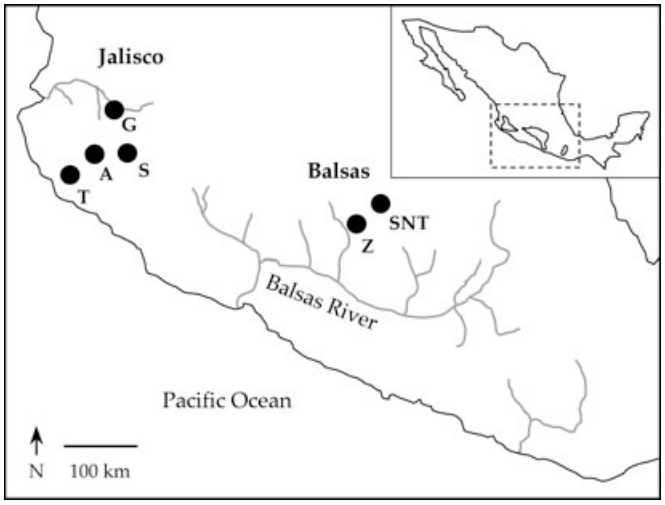

Figure 1. Map of *Zea mays* ssp. *parviglumis* study populations in southwest Mexico; the inset map shows the current distribution of the entire subspecies.

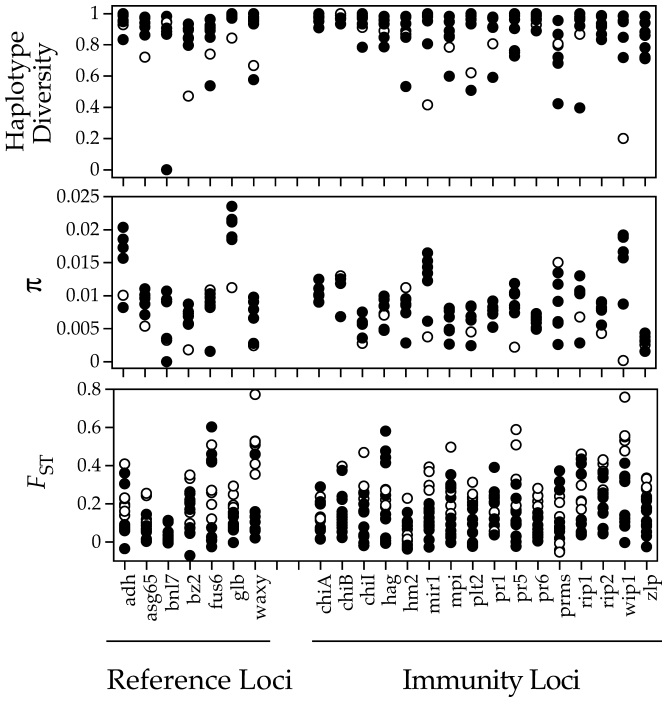

Figure 2. Population-specific estimates of haplotype diversity, nucleotide diversity (π), and population genetic differentiation (pairwise F_{ST}) for each of the seven reference loci and the 16 immunity loci. Estimates for population T are highlighted by open circles whereas the remaining populations are shown with filled circles.

plants in each of the six populations. One seed per maternal plant was germinated, grown to the seedling stage, and leaves were harvested for DNA extraction using Dneasy plant kits (Qiagen, Valencia, CA). We sampled 16 immunity loci and seven reference (nonimmunity) loci that have no known role in plant resistance; all loci were sampled from the same DNAs. Five of these reference loci include protein-coding regions with diverse function in plant growth and development and two loci are noncoding (Table 1). An analysis of nucleotide polymorphism and population structure for five of these reference loci has been reported previously (Moeller et al. 2007). Data from the remaining 18 loci have not been reported elsewhere. All sequences have been submitted to GenBank (Table 1).

CHARACTERIZATION OF SEQUENCE VARIATION

We used PCR to amplify the 23 immunity and reference loci from each of 84 DNAs (Table 1). PCR products were either directly sequenced or more often cloned into pGEM-T vectors (Promega, Madison, WI) before sequencing. For each DNA sample, one cloned fragment per locus was sequenced. Sequences were assembled and aligned in BioEdit 7.0.4.1 (Hall 1999). When singleton variants from cloned fragments were identified in alignments, they were checked by directly sequencing PCR products or by sequencing multiple clones from multiple PCR reactions. Sequenced regions ranged in size from 492 bp (*pr1*) to 2536 bp (*hm2*) (Table 1). For each population sample for each locus, we used DNAsp 4.0 (Rozas et al. 2003) to calculated six commonly used estimators of sequence variation: haplotype diversity (H_d, calculated using eq. 8.4, in Nei 1987), nucleotide diversity (π, Nei 1987), Tajima's *D* (Tajima 1989), population genetic differentiation from other populations (F_{ST}, based on genetic distance), population recombination rate (4Nr scaled by π) (Hudson 1987), and the relationship between within-species diversity and between-species divergence (for which we used *Tripsacum dactyloides* (Poaceae) as an outgroup). For estimates of population genetic differentiation, we found that values of F_{ST}, a statistic based on diversity within and between populations, were strongly correlated with estimates of S_{nn}, a statistic based on an allele's nearest-neighbor in a coalescent framework (Hudson 2000). Because the values of these two statistics were highly correlated and produced qualitatively similar results, we present only estimates of F_{ST}. All descriptors of diversity and divergence were estimated using DNAsp 4.0 (Rozas et al. 2003) and F_{ST} estimates were made using Arlequin (Schneider et al. 2000)

STATISTICAL ANALYSES

To test for geographic variation in the evolutionary history of immunity genes, we took three analytical approaches. Our first approach was to test for differences in nucleotide diversity and population structure (H_d, π, π_{rep}/ π_{sil}, Tajima's *D*, and F_{ST}) between the set of immunity and reference loci for both range-wide and population-specific samples. For range-wide samples, we compared gene classes using nonparametric Wilcoxon rank sum tests. For population-specific samples, we used the same statistical tests but conducted separate analyses for each population. These analyses test whether polymorphism and population

structure at immunity loci deviate consistently from that of other loci in the genome. If immunity loci are typically the subject of geographically variable selection, then we expect significantly lower sequence variation (H_d and π), frequency spectra of polymorphism more strongly skewed toward rare variations (Tajima's D), and higher F_{ST} at immunity relative to reference loci. If alternative alleles are favored by selection in different populations, then we expect an elevated ratio of π_{rep} / π_{sil} in range-wide samples of immunity loci, but not in population-specific samples. These tests for nonneutral evolution of immunity loci are conservative given that a significant difference between immunity and reference loci is expected only if the effects of selection are consistent across immunity loci. Moreover, if nonneutral evolution in the candidate (immunity) loci is not common, significant differences will not likely be detected.

Our second approach to testing for local adaptation in immunity genes was to use a maximum-likelihood HKA test (mlHKA, Wright and Charlesworth 2004), which examines the relationship between within-species nucleotide polymorphism and between-species sequence divergence for a set of candidate loci and reference loci (our analyses use sequence data from *T. dactyloides* as an outgroup). The test uses maximum likelihood to determine whether models allowing the selection parameter, k, to vary freely for immunity genes (but where reference loci are fixed to evolve neutrally, $k = 1$) provide a significantly improved fit over completely neutral models where k is fixed at 1 for all loci. We conducted separate mlHKA tests for each population to evaluate whether the evolutionary history of immunity genes has differed among populations, consistent with geographically variable selection. From each analysis, we examined estimates of the selection parameter, k, to determine whether polymorphism was elevated or reduced relative to neutral expectations, suggestive of balancing versus directional selection, respectively. For individual loci that exhibited strong deviations from neutral expectations in the selection models ($k > 2$ or $k < 0.5$), we conducted separate analyses allowing for k to vary freely only for the deviant locus.

Our third approach tested whether individual immunity loci have extreme patterns of sequence variation and population structure in comparison to (1) distributions of population genetic parameters based on coalescent simulations of subdivided populations and (2) empirical distributions of population genetic parameters based on the reference loci sampled in this study. As described above, unusually high F_{ST} and low sequence variation within populations may indicate geographically variable selection. Values for immunity loci in the lower 5% of distributions for nucleotide diversity and the upper 5% of the distributions for F_{ST} were considered candidates for local adaptation. Coalescent simulations of subdivided populations were conducted using Serial SIMCOAL (Anderson et al. 2005). Serial SIMCOAL, like SIMCOAL (Excoffier et al. 2000) upon which it is based, provides a framework for conducting coalescent simulations with multiple populations connected by gene flow and undergoing population growth or shrinkage. Population size and growth rate of each population, and gene flow between populations can all vary. We simulated six populations with initial population sizes of either 150,000 individuals (for two populations) or 250,000 individuals (for four populations), 1000 bp loci, and per locus mutation rate of $\mu = 10^{-6}$. These estimates of population size, rates of population growth, and rates of gene flow between pairs of populations were parameterized according to the range of estimates obtained from LAMARC (Kuhner 2006) using data from five of the reference loci analyzed previously (Moeller et al. 2007). LAMARC assumes that migration rates are at equilibrium and therefore that population structure has been constant. Because these assumptions are likely violated in *parviglumis* and most other species, migration rates may be biased (e.g., Moeller et al. 2007). However, the median and mode of the simulated distributions were very similar to that of our empirical distributions for each of the parameters examined (Figs. 3 and 4), suggesting that the simulations captured patterns of diversity found in our populations. Because simulation runs using different migration and population growth parameters produced qualitatively similar results, we report the simulation results from the run that provided the most conservative test for geographically variable selection. We calculated pairwise F_{ST}s and nucleotide diversity for each population for each of the 1000 simulated datasets using Arlequin (Schneider et al. 2000).

Results

RANGE-WIDE PATTERNS OF POLYMORPHISM AND POPULATION STRUCTURE

Our survey of 23 loci ($\sim$ 25,000 bases) from an average of 78 individuals/locus identified 1355 polymorphic sites (Table 1). Our range-wide samples harbored high haplotype diversity at both immunity and reference loci ($H_d > 0.9$ for 14 of the 16 immunity loci and five of six reference loci, Wilcoxon rank sum test: $Z = -0.201$, $P = 0.841$; Table 1). Range-wide nucleotide polymorphism (π) differed fourfold across immunity loci with *wip1* having the highest and *zlp* the lowest polymorphism (π = 0.0157 vs. 0.0038) and threefold among the reference loci; however, we found no significant difference in mean polymorphism between classes ($Z = 1.205$, $P = 0.228$). The frequency spectrum of polymorphism, as revealed by Tajima's D, also did not differ significantly between classes ($Z = 0.802$, $P = 0.423$). As with patterns of nucleotide diversity, the magnitude of population structure (global F_{ST} across all populations) did not differ significantly between immunity and reference loci ($Z = 0.367$, $P = 0.713$). The estimates of population differentiation at individual immunity loci differed almost sevenfold with *wip1* having the highest and *hm2* the lowest levels of F_{ST} (0.316 vs. 0.046;

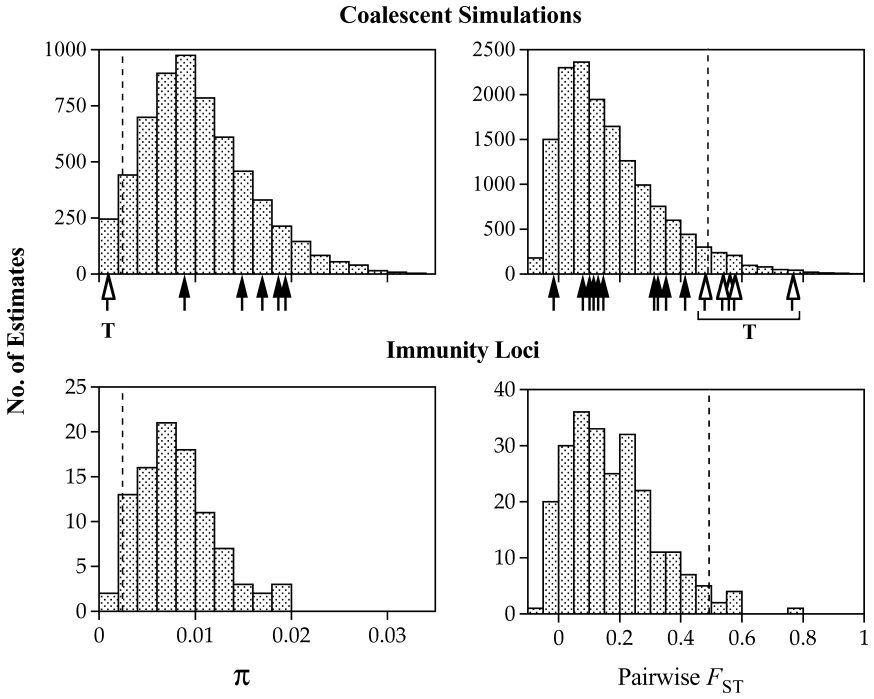

Figure 3. Distributions of nucleotide diversity (π) and population genetic differentiation (pairwise F_{ST}) derived from coalescent simulations of subdivided populations (top panels) along with distributions of observed population-specific estimates of π and F_{ST} for each of the immunity loci (bottom panels). Dashed lines indicate the lower 5% significance threshold for π and the upper 5% significance threshold for F_{ST}. Arrows show the values of each population for *wip1*; values for population T are highlighted using open arrows. The identity of outlier immunity loci is described in the *Results*.

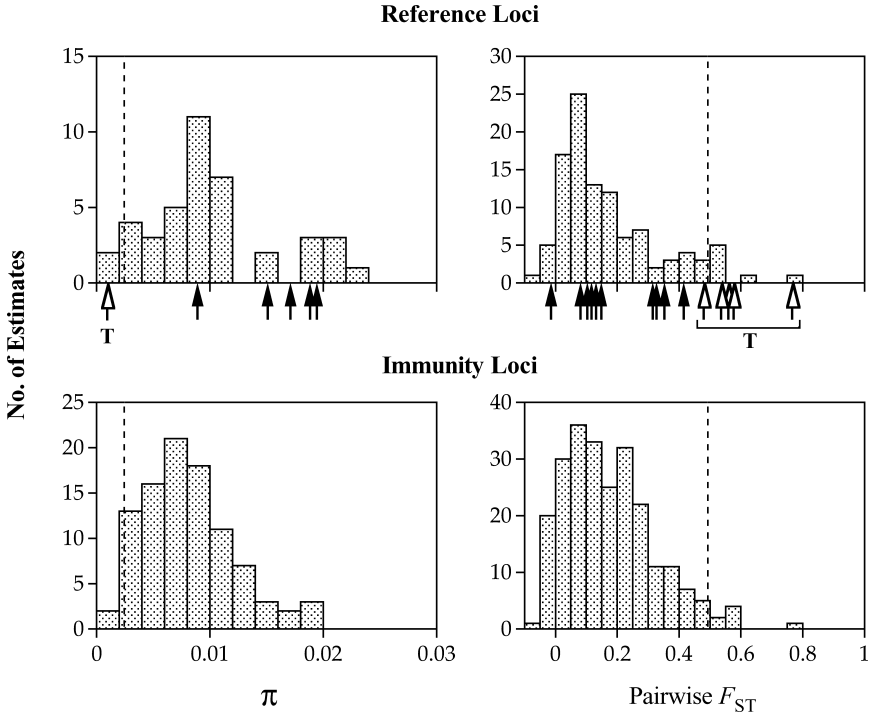

Figure 4. Distributions of nucleotide diversity (π) and population genetic differentiation (pairwise F_{ST}) from population-specific estimates for each of the reference loci (top panels) along with distributions of observed population-specific estimates of π and F_{ST} for each of the immunity loci (bottom panels). Dashed lines indicate the lower 5% significance threshold for π and the upper 5% significance threshold for F_{ST}. Arrows show the values of each population for *wip1*; values for population T are highlighted using open arrows. The identity of outlier immunity loci is described in the *Results*.

Table 1); this variance was greater than the fourfold range of F_{ST} values at reference loci. The one significant difference between immunity and reference loci that we detected was in the ratio of replacement to silent polymorphism, which was higher in immunity than reference loci ($Z = -2.35$, $P = 0.018$). This excess of replacement polymorphisms is consistent with findings from a smaller range-wide sample of *parviglumis* (Moeller and Tiffin 2005) and may be due to different alleles having been selected in different populations, that is geographically variable selection.

POPULATION-SPECIFIC PATTERNS OF POLYMORPHISM AND POPULATION STRUCTURE

Population-specific estimates of haplotype and nucleotide diversity tended to be lower than range-wide estimates, but comparisons between gene classes yielded similar patterns to those found in the range-wide samples. As with range-wide estimates, we did not find significant differences between immunity and reference loci for haplotype diversity (Wilcoxon rank sum test: $P > 0.61$ for all tests), nucleotide diversity ($P > 0.08$ for all tests), Tajima's D ($P > 0.07$ for all tests), or pairwise F_{ST} ($P > 0.36$ for all tests) within any of the six populations (Fig. 2). The ratio of replacement to silent polymorphism was significantly higher in immunity than reference loci in two of the six populations, G and SNT ($P = 0.01$ and $P = 0.05$, respectively), a pattern also found in the range-wide sample. In summary, our comparison of immunity to reference loci revealed no indication that immunity genes, as a class, are frequently targets of geographically variable selection. Instead, we found wide variation in the evolutionary history of loci in both classes.

MAXIMUM-LIKELIHOOD HKA TESTS WITHIN POPULATIONS

Maximum-likelihood HKA tests revealed no evidence that a model allowing for selection on all immunity genes provided a significantly improved fit over a completely neutral model for any population or across all populations combined (Table 2). Testing all immunity loci together, however, may mask evidence of selection that has acted on individual loci. Separate mlHKA tests for each of the loci that deviated strongly from neutral expectations in the initial analyses ($k > 2$ or $k < 0.05$, Table 2) identified three loci that deviated significantly from expected patterns under neutrality: polymorphism was strongly reduced for *wip1* in population T ($\chi^2 = 5.52$, df $= 1$, $P < 0.01$), and polymorphism was significantly elevated for *hag* in population SNT ($\chi^2 = 3.97$, df $= 1$, $P < 0.05$), and for *pr1* in population G ($\chi^2 = 3.48$, df $= 1$, $P < 0.05$). The remaining tests for individual loci (*hag* in T, *pr1* in S, SNT, and T; *mpi* in T, *chiA* in S, and *chiB* in T) did not indicate significant differences between neutral and selection models. As with comparisons between immunity and reference loci for diversity and F_{ST}, this analysis showed that there was considerable variation in the evolutionary history of the genes examined, with the majority of genes having patterns of diversity

Table 2. Maximum-likelihood HKA tests including seven immunity and seven reference loci for each of the six *parviglumis* populations and the entire range-wide sample. In the upper panel, log-likelihood values are shown from a completely neutral model and from a model allowing for the selection parameter, *k*, to vary freely for immunity loci; we did not find a significantly better fit for selection models compared to neutral models for any population. The lower panel shows the selection parameter, *k*, for each immunity gene from selection models. When values of *k* exceeded 2 or were less than 0.5 (shown in bold), an additional mlHKA test was run allowing for selection only on the locus deviating from neutral expectations. Asterisks denote the cases in which a neutral model could be rejected with **P*<0.05 or ***P*<0.01, and for these loci, the log likelihoods are also shown.

	Populations						Species-wide
	A	G	S	SNT	T	Z	
Neutral model (ln *L*)	−80.09	−80.76	−77.85	−83.50	−74.84	−81.84	−92.13
Selection model (ln *L*)	−80.66	−78.96	−77.85	−81.41	−70.04	−82.00	−93.10
Immunity Genes		*k* values					
hag	0.85	1.86	1.79	**4.78* (−80.66)**	**2.02**	1.38	1.49
pr1	1.82	**2.91* (−79.02)**	**3.81**	**3.13**	**4.58**	1.75	**2.66**
mpi	0.59	0.93	0.70	0.54	**2.59**	0.87	0.92
chiA	0.81	0.83	**2.01**	0.94	1.39	0.83	1.26
chiB	0.83	1.16	1.48	1.17	**2.44**	1.23	0.76
rip1	1.72	1.74	0.96	1.34	1.93	1.14	1.27
wip1	1.80	1.12	1.18	0.52	**0.00** (−72.08)**	1.18	1.11

indistinguishable from the reference loci, but with three immunity genes deviating from neutral expectations in one of the populations.

TESTS USING COALESCENT AND EMPIRICAL DISTRIBUTIONS

To further test whether individual immunity loci have been the targets of geographically variable selection, we compared population-specific estimates of π and pairwise F_{ST} to distributions based on (1) coalescent simulations of structured, growing populations (hereafter "coalescent distribution") and (2) the collection of population-specific estimates of parameters from the reference loci (hereafter "empirical distribution"). For nucleotide diversity (π), two immunity loci were in the lower tail of the coalescent distribution as well as the empirical distribution (*wip1*, popn T $P = 0.0002$; *zlp*, popn SNT $P = 0.00155$). One other value fell into the lower 5% of the coalescent but not the empirical distribution (*pr5* popn T, $\pi = 0.0022$). Of these three loci, only *wip1* in population T was also identified as having unusually low nucleotide polymorphism in the mlHKA analyses (see above).

Values of F_{ST} for seven pairwise comparisons between populations (involving three immunity genes) were in the upper tail of both the coalescent and empirical distributions, indicating unusually high population structure for these genes. Although this frequency of extreme values is approximately what would be expected by chance, four of these values were for *wip1* and involved comparisons between population T and other populations ($F_{ST} = 0.53 - 0.76$). A fifth F_{ST} value for *wip1* involving population T (and G) was in the upper 6% of both the coalescent and empirical distributions (*wip1*, G vs. T, $F_{ST} = 0.48$). Of the remaining extreme F_{ST} values, two were for *pr5* and involved comparisons between population T and two other populations (G and S, $F_{ST} = 0.59$ and 0.51). The remaining significant value was for *hag* and involved a comparison between populations A and S ($F_{ST} = 0.58$). The F_{ST} for *pr5* between populations S and T was in the upper tail of the coalescent but not the empirical distribution. Comparisons between immunity loci and either coalescent or empirical distributions were remarkably similar, with the empirical distributions identifying slightly fewer extreme values for immunity loci.

Across all tests, only *wip1* showed strong evidence for geographically variable selection. For *wip1*, we found very low nucleotide diversity and only two haplotypes in population T (with one haplotype represented by a single sequence and differing from the other at a single silent site in the flanking region); whereas, nucleotide diversity (π) was between 51- and 112-fold higher in the remaining five populations (see Fig. 5). Most notably *wip1* alleles in population T differed from nearly all other *wip1* alleles by a 565-bp insertion in the 3′ flanking region and a replacement substitution in the active site of the *wip1* protein. These patterns of nucleotide variation resulted in unusually high estimates of genetic differentiation (F_{ST}) at *wip1* between population T and other populations; whereas genetic differentiation for *wip1* between other pairs of populations (excluding T) were not unusually high or approaching significance.

Discussion

GEOGRAPHIC VARIATION IN SELECTION AT THE MOLECULAR LEVEL

Species interactions and patterns of selection on ecologically important traits frequently vary among geographic locations. The extent to which local selection shapes nucleotide diversity at the genes that mediate these interactions, however, is not well understood. If spatial variation in selection has shaped patterns of variation at the molecular level, then we expect population structure and nucleotide diversity will differ significantly between genes subject to local selection and those that are not subject to local selection. Alternatively, if the spatial pattern and nature of selection have been weak or unstable through time, then geographic variation in selection may leave no clear signature. In this study we searched for evidence of local adaptation at genes involved in protecting plants against pathogens and herbivores. This problem is of particular interest for defense genes because evolutionary ecologists have documented that geographically distinct populations often harbor different defense phenotypes and experience different selection in contemporary populations.

Of the 16 immunity genes sampled from six *parviglumis* populations, we detected evidence for geographically variable selection for only one immunity gene, the wound-inducible serine protease inhibitor (*wip1*). Like many other protease inhibitors, *wip1* confers plant resistance to insect herbivores by inhibiting chymotrypsin proteases in insect guts, and thus interferes with the hydrolysis of dietary proteins (Rohrmeier and Lehle 1993). Patterns of nucleotide variation within populations and divergence between populations both suggest a historical pattern of geographically variable selection on *wip1*. First, range-wide nucleotide polymorphism at *wip1* was the highest among all immunity loci, and five of the six populations harbored high levels of variation similar to that observed in the range-wide sample. By contrast, polymorphism at *wip1* in population T was over 50-fold lower than the remaining populations, had the lowest nucleotide diversity of all population samples of immunity genes, and was an outlier in both coalescent and empirical distributions based on reference loci. Maximum-likelihood HKA analyses confirmed that *wip1* had significantly reduced polymorphism in population T relative to other immunity and reference loci sampled in this same population and lower levels of polymorphism than expected in the absence of selection. These results suggest the action of directional selection on *wip1* within population T. Second, genetic

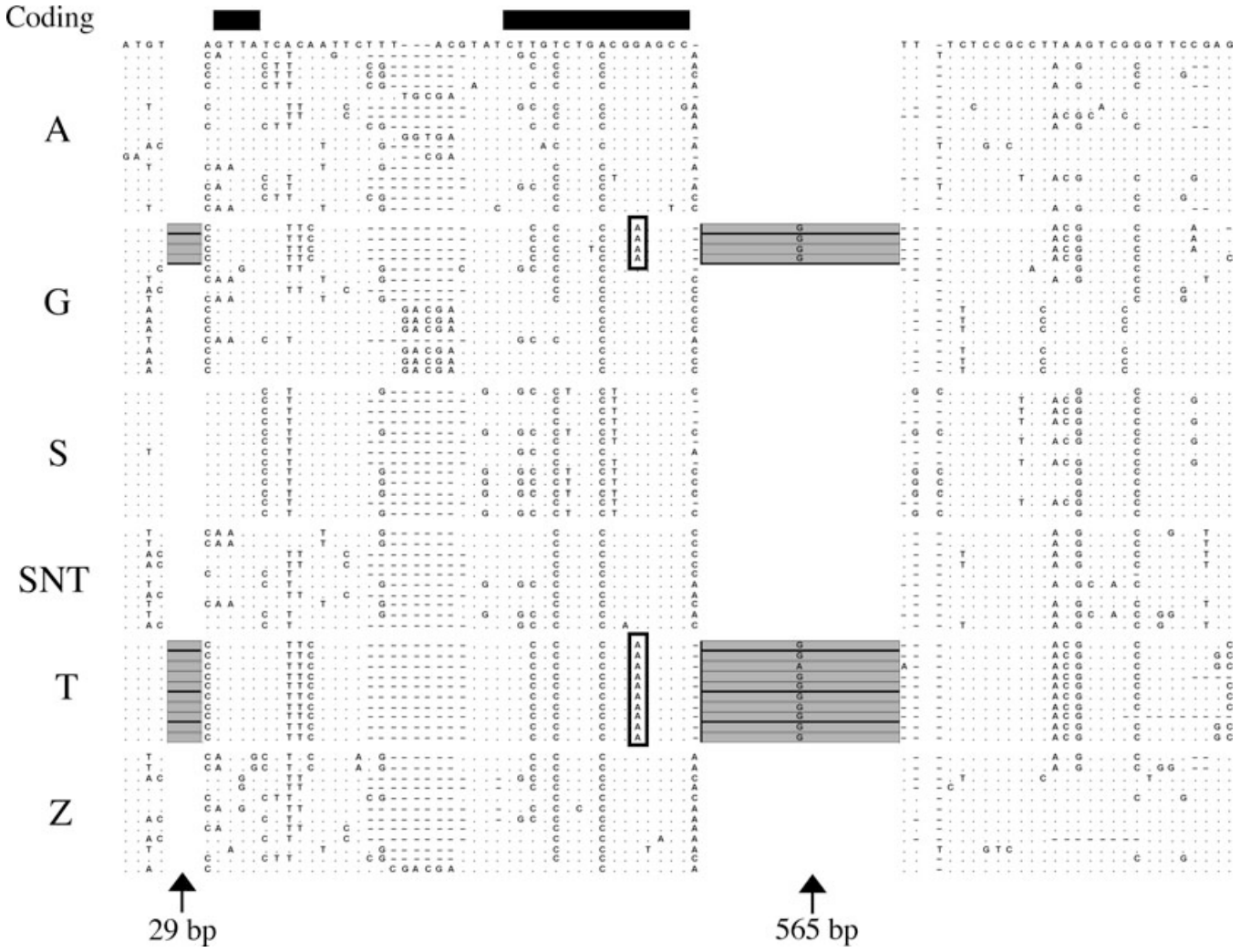

Figure 5. Gene structure and polymorphism in *wip1* for each of the six populations. The length of the aligned sequences is 1382 bp with one intron and 45 segregating sites. The two indels segregating in our population sample are shown with gray bars and the only replacement substitution in the active site of the protein is highlighted by black boxes.

differentiation (F_{ST}) at *wip1* between population T and other populations included some of the highest values measured and was significantly higher than predicted on the basis of coalescent simulations and the empirical distribution based on reference loci. Genetic differentiation at *wip1* between other pairs of populations (excluding T) was not unusually high or approaching significance. These results suggest that population structure at *wip1* differs strongly from that observed for other loci, consistent with geographically variable selection.

A closer examination of sequence variation in *wip1* showed that population T harbored a single segregating site across all 1205 bases; the remaining populations had 11–35 segregating sites (Fig. 5). Most notably, when compared to the remaining populations, all individuals in population T differed from nearly all other sequences by a 565 bp insertion in the 3′ flanking region of the gene and at a replacement substitution resulting in a nonpolar to polar amino acid change (glycine to serine) immediately adjacent to the active site of the protein (Fig. 4). The close proximity of this amino-acid change to the molecule's active site may alter the stereochemical fit between the *wip1* protein and chymotrypsin proteases, which in turn is expected to alter the efficacy of enzyme inhibition (Rohrmeier and Lehle 1993). Analyses of *wip1* divergence among grass lineages has also revealed evidence of adaptive evolution in the region of the protein that is near the active site (Tiffin and Gaut 2001). Interestingly, the population T allele is also found at low frequency in population G (Fig. 5). A previous analysis of five nuclear loci not involved in host defense revealed evidence for directional migration primarily from T to G (Moeller et al. 2007), suggesting that gene flow may have introduced this novel allele into population G.

Patterns of diversity at *wip1* suggest one of two possible evolutionary scenarios. One possibility is that these patterns reflect

local adaptation, either because natural selection favors a different allele in population T than in other populations or that *wip1* is under positive selection in population T but not in the other populations. Because population T is found at a lower elevation than the other populations we surveyed, historical biotic environments in this population may have differed from other sampled populations. Under this scenario, the population T allele may be found in population G because of recent migration but may be neutral or deleterious in that environment. A second possible explanation for the patterns of diversity at *wip1* is that the novel *wip1* allele found in population T confers an advantage across all populations but has yet to spread to fixation due to limited gene flow, particularly among Jalisco populations (Moeller et al. 2007). Under this scenario, the recent selective sweep in population T does not reflect geographically variable selection but rather the initial phase of an ongoing selective sweep. In structured populations, the time it takes for a favorable allele to spread to fixation will depend not only on the initial frequency and selective advantage, but also the time it takes for migration to move the allele across populations (Cherry and Wakeley 2003).

These two scenarios could be differentiated using field experiments if current biotic environments reflect the historical selective environments that drove differentiation. It is not clear, however, that this is a reasonable assumption. Herbivore and disease pressure are highly variable in nature (e.g., Root 1996) and therefore selection on plant defenses may be inconsistent through time. Moreover, the acquisition of novel plant defenses through adaptive evolution may reduce or eliminate enemy pressure in natural populations, leading to an important shift in the selective environment. Therefore, if the enemy pressure that caused selection on *wip1* has abated, then field studies in current environments may not represent the environment in which adaptation occurred. Both of these issues pose challenges for making connections between ecological process and evolutionary change at the molecular level.

Patterns of sequence variation in two other genes may be consistent with geographically variable selection, but the results are much weaker than those for *wip1*. We found significantly high levels of F_{ST} between population A and some of the remaining populations at *hag*, which encodes a thaumatin-like protein, along with evidence that the frequency spectrum of polymorphism harbored an excess of derived mutations in population A but no others (Fay and Wu's $H = -7.85$, $P < 0.05$). Similarly, for *pr5*, we found stronger than expected genetic differentiation between population T and a subset of other populations, along with nucleotide polymorphism that was significantly lower than expected ($\pi = 0.0022$, $P < 0.05$) and 4- to 5-fold less than that of the other populations. Regardless of whether patterns of sequence variation at these two genes are viewed as indicative of local adaptation, it is clear that we found little convincing support for geographically variable selection in the majority of immunity genes. In one case, patterns of sequence variation appear to conform to predictions under a range-wide selective sweep. For *hm2* we found unusually low levels of genetic divergence among populations, consistent with previous results indicating that *hm2* has been a target of positive selection in *parviglumis* (Zhang et al. 2002). Overall, these results suggest that species interactions in this system may not often be characterized by strong and consistent spatially variable patterns of selection, or that their imprint on the genome is too weak to detect by available methods.

EVALUATING EVIDENCE FOR GEOGRAPHICALLY VARIABLE SELECTION

To date, most evidence for geographically variable selection at the molecular level has come from studies of animals, particularly humans. For example, human populations have unusually high divergence (F_{ST} values) at genes influencing disease resistance (Tishkoff et al. 2001; Hamblin et al. 2002), lactose intolerance (Hollox et al. 2001), skin pigmentation (Rana et al. 1999), diabetes susceptibility (Fullerton et al. 2002), and behavior (Gilad et al. 2002). Most of these studies identified genes that were a priori predicted to show adaptive population differentiation due to observed differences in phenotypes; although more recent analyses have sought to use genome scans to identify a suite of loci that were not a priori suspected to be the targets of geographically variable selection (e.g., Akey et al. 2007). In some cases, extreme levels of population structure (F_{ST}) have been identified by comparing observed values to distributions of population genetic parameters derived from coalescent simulation (e.g., Bowcock et al. 1991). This model-based test for local selection has been criticized because simulation results are sensitive to variation in population demographic history. Incorporating information on migration structure and changes in population size, as we have done here, allow for more realistic simulation-based tests, compared to models that assume stable population size and panmixis (e.g., Storz et al. 2004). In our simulations, the median and mode of the coalescent-based distributions of nucleotide diversity and population differentiation was very similar to empirical distributions, suggesting that our model-based tests for local selection are unlikely to have been biased.

Indirect model-based tests for geographically variable selection are increasingly being replaced by the use of empirical distributions of population genetic parameters, particularly in model organisms where genome-wide polymorphism data are now available. For example, Akey et al. (2007) used an empirical distribution of F_{ST} based on SNP variation in humans to identify 156 candidate genes that may have been shaped by geographically variable selection. The application of this direct analytical approach remains challenging for nonmodel organisms, where high-density genome-wide polymorphism data are not yet available for

population samples. Although our study included relatively few reference genes, our tests involving empirical distributions based on our reference loci produced very similar results to tests involving coalescent distributions. The few studies to date that have detected nucleotide-level signatures of local adaptation in nonmodel organisms have compared spatial patterns of variation in candidate loci to spatial patterns in sets of unlinked loci (e.g., Hoekstra et al. 2004; Storz and Dubach 2004; Hemmer-Hansen et al. 2007; Storz et al. 2007) or examined genome-wide variation in anonymous markers such as AFLPs rather than sequence variation in candidate genes of known function (e.g., Emelianov et al. 2004; Egan et al. 2008). Although these approaches to testing for local adaptation at the molecular level have rarely been taken in studies of plants, one study identified candidate genes underlying adaptation to drought and salt tolerance in *Helianthus annuus* (Kane and Rieseberg 2007). A second study by deMeaux et al. (2003) did not find differences in population structure between plant resistance genes and RAPD markers, but found some inconsistency between patterns of population structure at the molecular level and in resistance phenotypes. As genome-wide polymorphism datasets become available for population samples, our ability to distinguish geographically variable selection from other phenomena such as demographic history will be greatly improved.

LIMITS TO DETECTING GEOGRAPHICALLY VARIABLE SELECTION

Kelly (2006) has recently shown that both biological and statistical factors may limit the ability to detect selection on genes influencing quantitative traits under geographically variable selection. Using simulations of sequence evolution in the flanking regions of QTL that underlie complex traits, Kelly (2006) showed that selection that favored different phenotypes in different populations strongly affected quantitative genetic variation but rarely affected nucleotide diversity in a manner that was detectable using standard tests of nonneutral evolution. Based on these results, it is likely that surveys of nucleotide diversity at genes that contribute to variation in polygenic traits, such as the one presented here, may underestimate the frequency with which phenotypes are the subjects of local selection. These results are also important because they suggest that local adaptation may often occur through standing variation, rather than being dependent on new mutations or alleles that enter populations through migration.

The immunity loci in our study are all known to be upregulated in response to and/or effective in defending against natural enemies, but the complexity of the genetics underlying phenotypes is not known for most of them. Even in cases in which a biochemical function can be assigned to a protein, we do not know how these functions are integrated into a phenotype upon which selection acts. It is likely that many of the genes included in our study (with the possible exception of *hm2*) contribute to a multilocus phenotype. In addition, these genes may be pleiotropic and confer resistance to multiple enemies, further complicating the likelihood of detecting local selection in gene sequences. This genetic architecture may have limited our ability to detect signatures of geographically variable selection at the molecular level, even if selection has been stable over many generations.

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