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Author(s): Yuri P. Springer

Source: *Evolution*, 61(8):1812-1822.

Published By: The Society for the Study of Evolution

DOI: <http://dx.doi.org/10.1111/j.1558-5646.2007.00156.x>

URL: <http://www.bioone.org/doi/full/10.1111/j.1558-5646.2007.00156.x>

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# CLINAL RESISTANCE STRUCTURE AND PATHOGEN LOCAL ADAPTATION IN A SERPENTINE FLAX–FLAX RUST INTERACTION

Yuri P. Springer

Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, A316 Earth and Marine Sciences Bldg., Santa Cruz, California 95064

E-mail: [yurispringer@gmail.com](mailto:yurispringer@gmail.com)

Received January 30, 2007

Accepted March 29, 2007

Because disease resistance is a hallmark signature of pathogen-mediated selection pressure on hosts, studies of resistance structure (the spatial distribution of disease resistance genes among conspecific host populations) can provide valuable insights into the influence of pathogens on host evolution and spatial variation in the magnitude of their effects. To date few studies of wild plant–pathogen interactions have characterized resistance structure by sampling across the host’s biogeographic range, and only a handful have paired such investigations with studies of disease levels under natural conditions. I used a greenhouse cross-inoculation experiment to characterize genetic resistance of 16 populations of California dwarf flax (*Hesperolinon californicum*) to attack by multiple samples of the rust fungus *Melampsora lini*. I documented a latitudinal cline in resistance structure, manifest across the host’s biogeographic range, which mirrored almost identically a cline in infection prevalence documented through field surveys of disease in study populations. These results provide empirical evidence for clinal patterns of antagonistic selection pressure, demonstrate that such patterns can be manifest across broad biogeographic scales, and suggest that rates of disease prevalence in wild plant populations may be tightly linked to the distribution of host resistance genes. Tests for local adaptation of the fungus revealed evidence of the phenomenon (significantly greater infection in sympatric plant–fungal pairings) as well as the potential for substantial bias to be introduced into statistical analyses by spatial patterns of host resistance structure.

**KEY WORDS:** Coevolution, *Hesperolinon californicum*, local adaptation, *Melampsora lini*, plant–pathogen interactions, resistance structure, serpentine soil.

Although the links between genetic resistance and the prevalence and severity of plant diseases have long been appreciated in agricultural settings (Browning and Frey 1969; Wolfe 1985; Garrett and Mundt 1999; Zhu et al. 2000) comparatively little is known about host resistance structure and its association with disease dynamics in natural plant–pathogen interactions (Burdon 1987a). A growing body of evidence suggests that the geographic distribution of resistance genes among populations of wild plants is often highly heterogeneous (Hunt and Van Sickle 1984; Parker 1985; Burdon 1987b; Jarosz and Burdon 1991; Bevan et al. 1993; Antonovics et al. 1994; Espiau et al. 1998; Burdon et al. 1999; Ericson et al. 2002; Laine 2004), but many studies of resistance

structure are modest in their spatial breadth, considering only a handful of host populations or confined to a small fraction of the host’s biogeographic range. Essentially nothing is known about associations between genetic resistance and spatial patterns of disease in natural systems because only a handful of studies have coupled investigations of resistance structure and epidemiology in the same interaction (Alexander et al. 1996; Carlsson-Graner 1997; Thrall and Burdon 2000; Laine 2004). Such studies are critical for characterizing and linking the ecological and coevolutionary dynamics of plant–pathogen interactions in natural ecosystems.

Because of their often-intimate associations and strong reciprocal fitness effects, plants and their pathogens represent model

systems in which to study the process of coevolution and the demographic, genetic, and biogeographic patterns that result. The geographic mosaic theory of coevolution posits that spatial variation in the frequency and intensity of reciprocal selection among geographically discrete, interacting populations can generate a mosaic of coevolutionary dynamics and outcomes for a given interaction (Thompson 1994, 1999a,b). This local context dependence is the result of geographic heterogeneity in characteristics of the focal species (e.g., demography, life-history traits, genetic diversity) and in biotic and abiotic features of their external environment. Reciprocal selection will be strong in some populations (“hot spots”) but weak or nonexistent in others (“cold spots”), and selection may favor the evolution of different traits in different locations (Gomulkiewicz et al. 2000). Local coevolutionary signatures can be further modified by stochastic events (e.g., genetic drift) and exchange between populations (e.g., gene flow, metapopulation dynamics). As a result of this combination of factors, traits important for coevolutionary interactions are expected to exhibit significant geographic structure, evolving at different rates and along varied evolutionary trajectories in different populations.

As genetic resistance to infection is a trait central to host–pathogen coevolution, studies of spatial patterns of resistance structure are implicitly characterizations of coevolutionary mosaics. Genes that confer resistance in host plants are often highly specific in terms of the pathogens that activate them and the defensive benefits that their expression confers (Staskawicz et al. 1995; Hammond-Kosack and Jones 1997; Ellis et al. 2000). Aside from stochastic events and selection that may be imposed by costs of resistance (Bergelson and Purrington 1996; Brown 2002; Tian et al. 2003) or pleiotropy (Parker 1990), the maintenance and distribution of these genes should be influenced primarily by pathogen-imposed selection. Theory predicts that levels of genetic resistance should be inversely related to the frequency and/or severity of infection (Haldane 1949; Frank 1993), and that for local interacting populations the two parameters should cycle out of phase as a result of time-lagged, frequency-dependent selection associated with host–pathogen interactions (Hamilton et al. 1990). Pairing studies of resistance structure and epidemiology in the same system provides information on the overall influence of pathogen-mediated selection on host resistance and on spatial variation in the strength of reciprocal selection among different coevolving populations.

Here I present the results of a cross-inoculation experiment in which California dwarf flax (*Hesperolinon californicum*) from 16 populations spanning the host’s biogeographic range were challenged with multiple samples of the fungal rust *Melampsora lini*. I used the infection data generated by the experiment to characterize host resistance structure, and by pairing these findings with data on disease prevalence collected in the field (Y. P. Springer, un-

publ. ms.), assessed the concordance between resistance levels of host populations and rates of rust infection in the wild. The design of the inoculation experiment allowed me to test for a system-level signature of pathogen local adaptation, and because of the unique spatial distribution of resistance genes among *H. californicum* populations, the results provide an insightful example of potential confounding effects of host resistance structure on such analyses.

## Methods

### HOSTS AND PATHOGEN

California dwarf flax, *H. californicum* Small (Linaceae), is a diminutive annual, generally 20- to 40-cm tall at flowering, with thin stems and leaves (McCarten 1993). The species is endemic to California, growing primarily in the Coast Range Mountains north of San Francisco Bay (Sharsmith 1961). The genus *Hesperolinon* contains 13 species, all of which are associated to some degree with serpentine soils, and has its center of diversity in the Napa/Lake county region of California where serpentine outcrops exist in abundance (Sharsmith 1961). *Melampsora lini* Persoon is a macrocyclic, wind dispersed, autecious rust fungus (Uredinales) that forms urediospores in pustules on the stems and leaves of infected plants (Flor 1954). It is specific to hosts in the family Linaceae and although infection of 12 of the 13 species of *Hesperolinon* has been observed it is unclear whether species-specific strains of *M. lini* have evolved within the host genus (Y. P. Springer, pers. obs.). As with most rusts, infections are non-systemic and result in loss of plant vigor or death via destruction of photosynthetic tissue and increased desiccation through damaged cuticle surfaces (Littlefield 1981). Field studies on the fitness costs of rust infection experienced by *H. californicum* have documented significant reductions in both seedling survival and adult fecundity. As an example, when compared with healthy conspecifics, moderate to severely infected individuals (> 30% of photosynthetic tissue covered by pustules) had on average a 32% higher rate of mortality as seedlings (6.7% vs. 38.7%,  $\chi^2 = 304.67$ ,  $df = 1$ ,  $P < 0.0001$ ,  $n = 2063$  total observations of 852 plants) and produced almost one-eighth the number of viable seed capsules as adults (mean  $\pm$  SE:  $21.0 \pm 1.8$  vs.  $2.8 \pm 0.94$ ,  $F_{1,138} = 76.29$ ,  $P < 0.0001$ ) (Y. P. Springer, unpubl. ms.). Because *M. lini* is an obligate biotroph, rust populations experience dramatic seasonal bottlenecks when hosts die back at the end of the growing season. The fungus overwinters as resting teliospores that reinfect their source population or are dispersed by wind to neighboring host populations in the subsequent year.

Research on interactions between *M. lini* and species of flax in the genus *Linum* has shown that resistance and virulence properties are governed by a gene-for-gene mechanism. This has been demonstrated in both agricultural settings (*L. usitatissimum*, [Flor 1955, 1956]) and wild host populations (*L. marginale*,

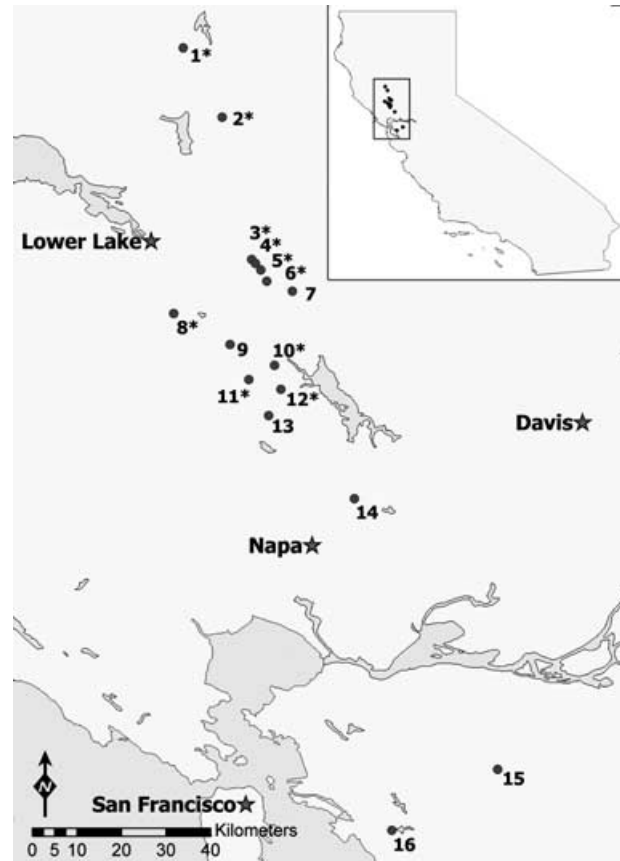
[Jarosz and Burdon 1991, 1992]). In gene-for-gene systems, genes determining resistance in the host interact with specific, corresponding avirulence genes in the pathogen, and resistant reactions only occur when host and pathogen strains are genetically matched (Crute et al. 1997). In cases of mismatching, the host lacks the resistance gene(s) necessary to recognize the avirulence gene(s) of the pathogen, and infection results. Gene-for-gene interactions have been putatively identified in a variety of crop species and in a growing number of interactions between wild plants and their pests in natural communities (Thompson and Burdon 1992). Given the close phylogenetic relationships between *H. californicum* and flax in the genus *Linum* (Y. P. Springer, unpubl. ms.), and the fact that they share a common pathogen, it seems likely that resistance and virulence in the *H. californicum*–*M. lini* interaction are also governed by a gene-for-gene mechanism. Although interpretation of experimental results are premised on this assumption, methods used to score infection are equally well suited to characterize resistance and virulence based on monogenic and polygenic systems.

#### CHARACTERIZING STUDY POPULATIONS

I determined the biogeographic range of *H. californicum* using a monograph on the genus *Hesperolinon* (Sharsmith 1961), herbarium records at the University of California Berkeley Jepson Herbarium ([www.calflora.org](http://www.calflora.org)), and interviews with local plant ecologists. I selected 16 study populations that collectively span the latitudinal extent of this range as completely as possible (Fig. 1). Populations are separated from each other by at least 1 km and range in size from roughly 1000 to more than 20,000 individuals. As part of another study, epidemiological surveys were conducted in a subset of these populations in 2001, and at all sites in 2002, 2003, and 2004 (Y. P. Springer, unpubl. ms.). Surveys were based on a “fixed-plot” approach using uniformly spaced transects and 0.25-m<sup>2</sup> quadrats. For each plant examined I visually estimated infection severity (percent of host leaf and stem tissue covered with rust pustules) using a modified James scale (James 1971) and assigned the plant to one of nine infection severity categories: 0% (uninfected), 1%, 5%, 10%, 25%, 50%, 70%, 90%, and 100%. Using these data I quantified disease prevalence (percent of hosts infected) and mean infection severity of each survey population. Results indicated that these two parameters were highly correlated. A more detailed discussion of these methods and results is provided elsewhere (Y. P. Springer, unpubl. ms.).

#### SAMPLE COLLECTION

I collected seeds in each *H. californicum* population following annual surveys in 2002. To balance sample size at 22 maternal lines per host population, seeds harvested in 2001 made up a percentage of the sample for populations 3 (8%), 4 (57%), 7 (100%) and 10 (5%). I collected *M. lini* spores from two *H. californicum*



**Figure 1.** Map showing locations and identification numbers of the 16 *H. californicum* study populations in northern California. Populations from which *M. lini* spores were collected are indicated symbolically (\*).

populations in 2003 and eight populations in 2004. For each of these 10 fungal samples, spores were harvested from multiple diseased plants in the field, pooled, and amplified in the laboratory on greenhouse grown plants from the same source population. To minimize the extent to which this process selected for particular fungal pathotypes, amplifications were completed after one fungal generation and used at least 30 host maternal lines collected across the entire spatial extent of the local host population. Three logistical complications precluded the generation of rust isolates using spores from a single source pustule as is customary protocol for inoculation experiments of this type. First, because of the extremely small size and low surface area of *H. californicum* leaves, amplification is relatively inefficient because inoculations produce a limited number of small pustules with low spore yields. Second, *M. lini* spores collected from *H. californicum* were unable to induce symptoms when inoculated onto 16 different agricultural strains of *L. usitatissimum* including the “universally susceptible” variety Hoshangabad used by Burdon and colleagues in their studies of *L. marginale*–*M. lini* interactions (Burdon and Jarosz 1991). Finally, attributes of host biology made it extremely difficult to keep *H. californicum* plants alive through multiple rounds of rust

amplification. I took two steps to attempt to control for the fact that amplification using spores from more than one pustules may have resulted in the inclusion of multiple rust pathotype in these fungal samples. First, for each sample, I collected spores from fewer than 10 diseased plants growing in very close proximity to one another in the field ( $\sim .5$  m radius). This spatially restricted sampling, performed early in the growing season when infection foci were few in number and spatially distinct, should have increased the likelihood that these plants were all infected by the same rust pathotype spread from a common local source of secondary inoculum. Second, I created extremely concentrated and homogeneously mixed inoculation solutions to insure that if multiple pathotypes had been amplified, each would be present at a high level of abundance, and each inoculated plant would have roughly the same chance of contacting spores of any given pathotype. In an attempt to maximize the number of unique pathotypes used in the experiment I focused sampling efforts among rather than within study sites, collecting one fungal sample per host population but gathering samples from multiple populations that span the latitudinal range over which rust infection of *H. californicum* occurs in the wild. Following separate amplification of each sample, spores were collected, lyophilized, and stored at  $-80^{\circ}\text{C}$ .

#### GREENHOUSE INOCULATION EXPERIMENT

I quantified levels of genetic resistance to rust infection present in the 16 study populations using a greenhouse inoculation experiment based on a replicate block design. Individual seedlings, collectively representing 352 maternal lines, were planted in separate 66-mL Pine cell Cone-tainers<sup>®</sup> (Stuwe and Sons, Corvallis, OR) and grown in the greenhouse (14-h day length,  $21^{\circ}\text{C}$  day/ $13^{\circ}\text{C}$  night) for 36 days. At this point the majority of plants were 3–6 cm tall and within the height range of plants in the field when rust infection is first observed. I haphazardly assigned one plant from each maternal line to each of the 11 inoculation blocks (10 fungal inoculations, one control), which consisted of two 200-cell capacity container storage racks, and to a position within that block. If fewer than 11 plants were available for a given maternal line I randomly assigned plants to inoculation blocks. On average, each block contained  $20.4 \pm 0.1$  (mean  $\pm$  SE) maternal lines per host population and  $326.1 \pm 1.5$  maternal lines overall.

I inoculated each block with one of the 10 rust samples. Lyophilized spores were thawed, rehydrated, and added to an 80-mL solution of autoclaved nanopure water with 0.05% Tween 20 surfactant (Acros Organics, NJ). Three 0.1-mL aliquots of each inoculation solution were used to estimate spore concentrations (using a hemacytometer) and viability (by scoring fungal germination tube formation on agar-coated microscope coverslips 24 h after solution preparation). I sprayed plants in each block to runoff with the assigned inoculation solution (water for the control block) using a Preval disposable aerosol sprayer (Precision Valve Corp,

New York) and then sealed blocks in separate humidity chambers at 100%RH for 24 h. Following this period plants were returned to the greenhouse. Resistance to infection was scored after 13 days using a categorical scale: 0 (fully incompatible reaction with no macroscopic, visible lesions), 1 (partially incompatible reaction with necrotic flecks and/or very small pustules on young leaves but no pustules on older leaves), 2 (partially incompatible reaction with almost full sized pustules on young leaves grading to fewer, smaller pustules or no pustules and/or necrotic flecks on older leaves), and 3 (fully compatible reaction characterized by large freely sporulating pustules and no signs of chlorosis on either young or old leaves). This scale was described by Burdon (1994), who showed that partially resistant phenotypes (1, 2), like their fully resistant and susceptible counterparts (0, 3) are controlled by single dominant genes in the *L. marginale*–*M. lini* interaction. The “infection score” recorded here is a proxy for pathogen “virulence” (defined as the ability of the rust to infect and induce disease symptoms in hosts) and host “resistance” (the infection score is inversely related to the degree of genetic resistance to rust infection). I also measured the height of each plant and included this variable in my analyses as a control for effects of plant size or surface area on infection. Previous work has shown that plant height is strongly correlated with number of leaves in *H. californicum* (Y. P. Springer, unpubl. data).

## Statistical Analyses

### QUANTIFYING RESISTANCE STRUCTURE AND LOCAL ADAPTATION

I analyzed infection data using a mixed model ANOVA in which the terms plant population, plant maternal line (nested within plant population), fungal population (the *H. californicum* source population for each fungal sample), plant height, allopatric versus sympatric (relative to the source population of plants and fungi in each inoculation pairing), and the interaction between plant population and fungal population were fit sequentially in the order listed. The interaction was decomposed into its component parts of a comparison between sympatric and allopatric combinations, which represented the diagonal versus off-diagonal elements in the matrix and is one measure of local adaptation (Gandon and Van Zandt 1998), and the residual interaction variance that represents the denominator for the test of the sympatric versus allopatric comparison. All factors were fixed except for plant maternal line, which was random. Infection score was the response variable in the model. Although categorical, this variable was treated as continuous in the analysis because it is ordinal and represents a continuously varying parameter (symptom expression). The main effects of plant and fungal population were significant, so I further decomposed these terms by comparing all pairwise combinations of least-squares means with *t*-tests and adjusting *P*-values



for multiple comparisons using the Tukey HSD procedure. I assessed concordance between field-measured disease prevalence and greenhouse-measured genetic resistance using linear regression of population means. All analyses were performed in JMP version 5.1.1 (SAS Institute, Cary, NC).

### EFFECTS OF RESISTANCE STRUCTURE ON TESTS OF LOCAL ADAPTATION

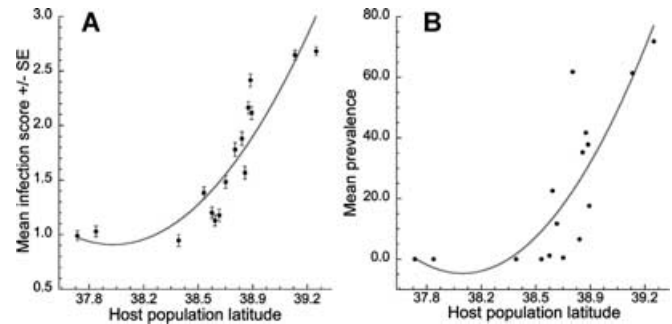
In their study of local adaptation in the *L. marginale*–*M. lini* system, Thrall et al. (2002) noted the potential for host resistance structure to bias tests of pathogen local adaptation based on results of cross inoculation experiments. In the present study, the particular geographic pattern of resistance structure provided an excellent heuristic example of this confounding effect. The direction a fungal sample was “moved” geographically to create an inoculation pairing had a marked influence on the relationship between infection and distance between host and pathogen populations, a commonly used measure of local adaptation (Gandon and Van Zandt 1998; Kaltz and Shykoff 1998). I visualized this effect by superimposing the results of three separate linear regressions of infection score versus distance between host and pathogen populations used for each inoculation pairing: I ran the analysis using the entire dataset and using the two subsets of data consisting of pairings that involved fungal samples “moved” either north or south. Mean infection scores were calculated for each plant population–fungal population combination, and inter-population distances, which ranged from one to 180 km, were quantified using ArcGIS 9.0 (ESRI, Redlands, CA).

I used an ANCOVA to quantitatively decouple the effects of host resistance structure and pathogen local adaptation on the relationship between infection and distance. In the model, mean infection score of each plant–fungal combination was the response variable and fungal population, plant population (a proxy for host resistance structure), distance between fungal population and plant population (a measure of pathogen local adaptation), and the fungal population by distance interaction term were explanatory variables. Distance was  $\log_{10}$ -transformed to meet the assumption of normality. I isolated and visualized the effect of distance predicted by this analysis using a plot of the partial residuals of infection versus  $\log_{10}$ -transformed distance. Finally, I quantified the relative contribution of local adaptation and host resistance structure to patterns of infection by comparing the adjusted  $r^2$  value of the full model with those of reduced models from which either the distance or plant population terms were excluded.

## Results

### QUANTIFYING RESISTANCE STRUCTURE AND LOCAL ADAPTATION

Results of the inoculation experiment indicated a latitudinal cline in genetic resistance across the biogeographic range of *H. cali-*



**Figure 2.** Greenhouse-measured genetic resistance (A) and field-measured disease prevalence (B) as a function of host population latitude. Mean infection score, which is inversely related to the degree of genetic resistance, was calculated by averaging within populations across all 10 greenhouse inoculations ( $r^2_{\text{adj}} = 0.80$ ,  $MS = 2.19$ ,  $F_{2,15} = 30.66$ ,  $P < 0.0001$ —Infection score =  $-61.68 + (1.63 \times \text{Latitude}) + 1.16 \times (\text{Latitude} - 38.66)^2$ ). Error bars denote one standard error. Mean prevalence was calculated by averaging within population across all field-survey years ( $r^2_{\text{adj}} = 0.64$ ,  $MS = 3294.56$ ,  $F_{2,15} = 14.14$ ,  $P = 0.0005$ —Mean Prevalence =  $-2456.51 + (63.93 \times \text{Latitude}) + 51.29 \times (\text{Latitude} - 38.66)^2$ ).

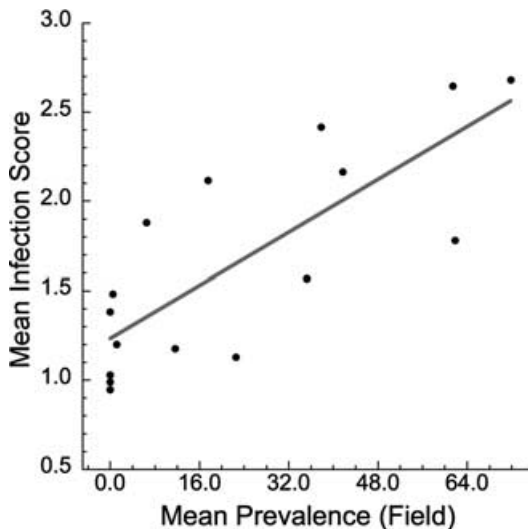
*fornicum* (Fig. 2A). Resistance was lowest in the north and increased gradually to the south (online Supplementary Table A1). This pattern mirrors almost identically the pattern of *M. lini* infection documented through epidemiological field surveys (Fig. 2B). Northern host populations that experienced the highest rates of rust infection in the wild had the lowest levels of genetic resistance. Moving south, the degree of genetic resistance to infection increased and rust prevalence rates declined. The highest levels of genetic resistance were detected in the southernmost host populations in which rust infection was never observed. Linear regression

**Table 1.** Variation in infection scores recorded in greenhouse inoculations as a function of plant and fungal population of origin, plant maternal line, plant height, and allopatric/sympatric nature of inoculation pairings.  $r^2_{\text{adj}} = 0.51$ ,  $MS = 3.694$ ,  $F_{479,3260} = 8.142$ ,  $P < 0.0001$ .

Source	df	MS	F
Plant population	15	71.21	156.96****
Plant maternal line (Plant Population)	319	1.27	2.81****
Fungal population	9	18.23	40.17****
Plant height	1	6.05	13.33***
Allopatric vs. sympatric <sup>1</sup>	1	9.90	11.57***
Plant population × fungal population	134	0.86	1.89****
Error	2781	0.45	

\*\*\*\* $P < 0.0001$ , \*\*\* $P < 0.001$ .

<sup>1</sup>Tested over the Plant Population × Fungal Population MS.



**Figure 3.** Mean greenhouse-measured infection score associated with each of the 16 host populations as a function of mean levels of disease prevalence associated with those populations in the field. Mean disease prevalence calculated by averaging yearly values across three to four years of epidemiological field surveys ( $r^2_{\text{adj}} = 0.59$ ,  $MS = 3.30$ ,  $F_{1,15} = 23.03$ ,  $P = 0.0003$ —Mean Infection Score =  $1.23 + 0.019 \times \text{Mean Prevalence (Field)}$ ).

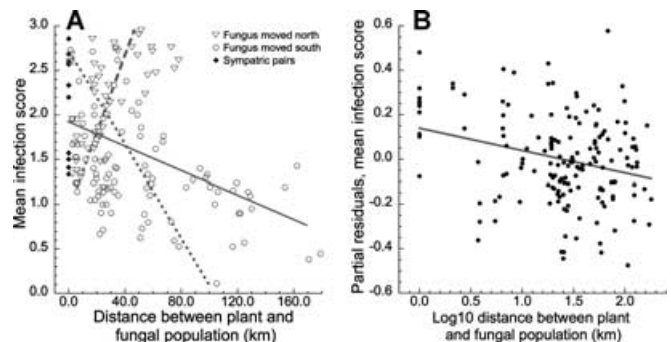
revealed that approximately 60% of the variation associated with field-measured disease prevalence could be explained by the level of genetic resistance (Fig. 3). Mean host infection score, averaged across all 16 populations, was  $1.66 \pm 0.15$  (mean  $\pm$  SE). Although there was significant variation in mean infection score among the ten fungal samples there was no associated latitudinal pattern (online Supplementary Fig. A1, Table A1). Additionally there was no relationship between the mean resistance of host populations and the mean virulence of associated fungal samples (online Supplementary Fig. A2). There was a significant positive relationship between height and infection score, but comparison of the adjusted  $r^2$  values of the full model with a model excluding height indicated that this effect accounted for less than 0.5% of the full model's predictive ability. Linear regression revealed no significant latitudinal trend in plant size. Overall, the effects included in the mixed model ANOVA explained 51% of observed variation in infection score data (Table 1). The average spore concentration of the inoculation solution aliquots (mean  $\pm$  SE) was  $3.6 \times 10^5 \pm 3.8 \times 10^4$  and spore germination rates were high (mean  $\pm$  SE =  $61.0 \pm 3.1\%$ ).

The significant effect of the allopatric/sympatric term in the ANOVA provided evidence for local adaptation of the pathogen at the system level. Significantly higher mean infection scores for sympatric plant–fungal pairings were produced by the analyses involving the entire dataset (mean  $\pm$  SE =  $2.09 \pm 0.06$  vs.  $1.64 \pm 0.02$ ,  $MS = 9.90$ ,  $F_{1,134} = 11.57$ ,  $P = 0.0009$ ) and data from the 10 host populations from which fungal samples had been

collected (mean  $\pm$  SE =  $2.09 \pm 0.06$  vs.  $1.85 \pm 0.02$ ,  $MS = 9.95$ ,  $F_{1,80} = 13.26$ ,  $P = 0.0005$ ). A one-tailed  $t$ -test confirmed that the global mean difference in infection scores between allopatric and sympatric pairings, calculated by averaging the absolute value of this difference calculated for each fungal sample, was significantly greater than zero (global mean difference = 0.63,  $t(9) = 5.03$ ,  $P = 0.0004$ ). Tests to detect pathogen local adaptation within individual host populations could not be performed due to the lack of replication of fungal samples at that scale. Multiple, single-pathotype fungal isolates from each study population would be necessary to quantify mean population fitness values of the pathogen at the scale of individual host populations.

### EFFECTS OF RESISTANCE STRUCTURE ON TESTS OF LOCAL ADAPTATION

The negative relationship between mean infection score and plant–fungal interpopulation distance produced by a linear regression using the entire dataset suggested a pronounced effect of pathogen local adaptation. Similar analyses involving subsets of the data coded for directionality of fungal “movement,” however, indicated that there was a positive relationship between mean infection score and distance when fungal samples were moved north (i.e., inoculated onto hosts from a higher latitude population) and a negative relationship when samples were moved south (Fig. 4A). These results demonstrated that distance and direction were confounded by the pattern of host resistance structure, a conclusion supported



**Figure 4.** Relationships between mean infection score for each plant population–fungal population inoculation pairing and their interpopulation distance. Panel A shows raw data with categorical designations for pairings involving fungal samples moved north (triangles, dashed line), fungal samples moved south (circles, dotted line), and sympatric plant–fungal pairs (diamonds). Solid gray regression line is fit to all data ( $r^2_{\text{adj}} = 0.15$ ,  $MS = 10.38$ ,  $F_{1,159} = 28.56$ ,  $P < 0.0001$ —Mean Infection Score +  $1.93 - (0.0065 \times \text{Distance})$ ). Panel B is a partial residuals plot illustrating effect of distance between plant and fungal population ( $\log_{10}$  transformed) on mean infection score after effects of direction have been removed by ANCOVA. ( $r^2_{\text{adj}} = 0.071$ ,  $MS = 0.48$ ,  $F_{1,159} = 13.13$ ,  $P = 0.0004$ —Partial Residuals Mean Infection Score =  $0.14 - (0.1 \times (\text{Log}_{10} \text{Distance} + 1))$ ).

**Table 2.** Results of ANCOVA examining the effects of fungal population, linear distance between plant population and fungal population, the distance  $\times$  fungal population interaction term, as well as plant population, on mean infection score of each plant-fungal pairing. Data transformations are indicated parenthetically.  $r^2_{\text{adj}} = 0.91$ ,  $MS = 1.846$ ,  $F_{34,159} = 46.04$ ,  $P < 0.0001$ .

Source	df	MS	F
Plant population	15	2.55	63.53****
Fungal population	9	0.68	17.00****
Distance between plant and fungal population ( $\log_{10+1}$ )	1	0.85	21.26****
Distance ( $\log_{10+1}$ ) $\times$ fungal population	9	0.04	1.11
Error	125	0.04	

\*\*\*\*  $P < 0.0001$ .

by the highly significant effect of plant population on infection in the ANCOVA model (Table 2). Results of the ANCOVA, however, indicated that there was an additional small but significant effect of distance unrelated to spatial patterns of host resistance (Table 2). Indeed, the regression of the partial residuals of mean infection score against distance produced a significant negative slope ( $r^2_{\text{adj}} = 0.071$ ,  $MS = 0.48$ ,  $F_{1,159} = 13.13$ ,  $P = 0.0004$ ) indicative of local adaptation (Fig. 4B). Comparing adjusted  $r^2$  values of the full ANCOVA model with reduced models in which single effects were excluded indicated that distance explained 2.0% of the variation in infection whereas plant population explained 84.4% and fungal population explained 13.5%. There was a significant effect of fungal population, presumably because of variation in virulence among the different samples, but there was no significant fungal population by distance interaction.

## Discussion

The inoculations performed to investigate resistance structure in *H. californicum* revealed a latitudinal cline in the distribution of rust resistance genes, with low resistance levels in northern host populations increasing gradually and continuously to high levels in the south. The likelihood that rust infection is mediated by a gene-for-gene mechanism means that the resistance phenotypes scored in the experiment are tightly linked to genes whose effects are highly specific and explicitly coevolutionary in nature. This result provides some of the clearest evidence to date of the existence of coevolutionary clines in natural ecosystems. Although the existence of such clines has been theoretically proposed (Thompson 1999a, 2005; Nuismer et al. 2000), most investigations of the spatial signatures of antagonistic coevolution have documented discrete patches, akin to Thompson's "hot spots" and "cold spots," where the effects of reciprocal selection are categorically defined as strong or weak (Davies and Brooke 1989a,b; Berenbaum and

Zangerl 1998; Benkman et al. 2001; Brodie and Ridenhour 2002; Thompson and Cunningham 2002; Fischer and Foitzik 2004). Empirical documentation of clinal antagonistic coevolution is limited to a handful of studies (Kraaijeveld and Godfray 1999; Krist et al. 2000; Toju and Sota 2006).

The cline in resistance levels among *H. californicum* populations mirrors almost identically the rates of *M. lini* infection experienced by those populations in the field, with high disease incidence in populations with low resistance. As part of related research (Y. P. Springer, unpubl. ms.), three to four years of annual epidemiological surveys conducted in the same 16 study populations documented a latitudinal cline in infection, with rust prevalence and infection disease high in northern host populations and decreasing gradually toward the south. There was no evidence for similar latitudinal patterns in host demography (population size, density), associated soil chemistry (abundance of 17 elemental analytes), or meteorological conditions known to be important for the rust development (temperature, relative humidity, free-water availability). Additionally, the present study failed to detect a latitudinal trend in genetic contributions to plant height. Taken together, these findings suggest that in northern regions of the host's range spatial patterns of infection may be determined in large part by host genetics. This relationship is more ambiguous in southern host populations because it is unclear whether lower infection rates are the result of lower pathogen abundance or greater host resistance. Given the dispersal ability of fungal rusts (Brown and Hovmøller 2002) and the observation of disease in congeneric host populations south of *H. californicum*'s southern range limit, it seems improbable that rust spores cannot reach these southerly *H. californicum* populations or that conditions there are inimical to fungal growth. Instead, rust spores are probably present but go undetected because of their inability to produce symptoms on highly resistant hosts. Negative feedback between host resistance and pathogen demography could generate lower infection rates by reducing the probability of primary infection and rates of local spread via secondary inoculum (Thrall and Jarosz 1994; Alexander et al. 1996; Thrall and Burdon 2000). Quantification of aerial spore concentrations would be needed to determine the relative contribution of host resistance and pathogen abundance to patterns of infection in southern *H. californicum* populations.

Although results of the inoculation experiment could not be used to quantify local adaptation at the scale of individual host populations, pooling information across the entire experiment indicated significantly greater infection in sympatric plant-fungal pairings relative to allopatric pairings at the "global" scale. This finding qualitatively parallels those generated by other cross-inoculation experiments (Parker 1985; Thrall et al. 2002; Laine 2005, but see Parker 1989; Kaltz et al. 1999, reviewed by Kaltz and Shykoff 1998; Lively et al. 2004) and is in agreement with theoretical predictions that local adaptation should arise when pathogen



virulence is relatively high (Lively 1999; Gandon 2002), and when migration rates of the pathogen are greater than those of the host (Gandon and Michalakis 2002; Morgan et al. 2005), criteria that are both met in this system. In conjunction with the clinal pattern of host resistance, regression analyses comparing infection scores with host–pathogen interpopulation distances produced a clear example of the potential confounding effects of host resistance structure on tests for local adaptation. Due to the cline in host resistance structure, fungal samples moved a given distance north always encountered less-resistant hosts and generated higher average infection scores, whereas the opposite result was produced by samples moved the same distance south. Because infection was never observed (and rust spores could not be collected) in the southern third of the host's biogeographic range, the longest distances “moved” by fungi in the experiment were to the south, onto highly resistant hosts that exhibited low infection. These datapoints acted to pull down the regression line in the global analysis, producing a negative slope and the impression of pronounced local adaptation. In contrast, results of the ANCOVA analysis and associated partial residuals regression that removed the effect of host resistance structure indicated that although significant, the effect of plant–fungal interpopulation distance on infection was considerably more subtle. Comparison of adjusted  $r^2$  values of reduced models indicated that the contribution of host resistance structure to infection was over 40 times greater than that of distance, a proxy for local adaptation.

Analyses of infection data were implicitly based on the assumption that each fungal sample contained a single pathogen isolate. Given the bulk sampling approach used to collect rust spores, however, it is possible that multiple pathotypes were present in one or more of the fungal samples. Under these conditions, inoculation results would tend to overestimate pathogen virulence because different fungal strains may possess different virulence factors. This effect would be problematic for comparisons of pathogen virulence across study systems but should not influence the within-system analyses presented here because they are based on relative rather than absolute virulence levels. Additionally, characterization of host resistance structure would have been confounded had different host populations shown the same levels of infection when challenged by a given fungal sample because this result could have been produced via infection by different pathotypes present in that sample. The pattern of continuous variation in infection severity among host populations observed in each of the 10 experimental inoculations largely assuages this concern because host populations clearly differed in resistance irrespective of whether fungal samples contained one or many pathotypes. It seems highly unlikely that infection by multiple pathotypes could have additively produced the qualitatively identical clinal pattern of infection observed in each of the inoculations. A more parsimonious explanation for this result is that some combination of

ecological and/or evolutionary forces has led to the accumulation of resistance genes in southern host populations and their absence in northern populations. Although unconventional for inoculation experiments of this type, the use of bulk sampling was compatible with the system-level focus of the study and was sufficient to characterize host resistance structure and detect a broad-scale signature of pathogen local adaptation.

Why southern host populations have such high levels of genetic resistance relative to northern populations is unclear. The observed negative relationship between host resistance and disease prevalence is likely evidence of asynchronous cycling of host resistance and pathogen infection generated by time-lagged, frequency-dependent selection (Jaenike 1978; Hamilton et al. 1990; Chaboudez and Burdon 1995). This hypothesis is premised on the assumption that host evolution and pathogen abundance are tightly linked by host demography, a likely scenario given the strong fitness costs of infection (Y. P. Springer, unpubl. ms.). The present distribution of resistance among southern host populations might be a residual evolutionary signal produced by greater historical disease pressure in this part of the host's range. Removal of susceptible genotypes by past infection would have reduced local host abundance and increased the relative frequency of resistant host genotypes. Both effects would gradually reduce pathogen population sizes, increasing the chances of local pathogen extinction and reducing the probability of subsequent recolonization. The host is primarily selfing and has water-dispersed seeds (Sharsmith 1961), so movement of resistance genes north via gene flow should be very slow. In contrast, the dispersal potential of windborne fungal spores is likely much greater, so pathogen virulence should exhibit less spatial structure than host resistance. These predictions are consistent with the fact that the same latitudinal cline in host resistance structure was observed in all 10 experimental inoculations, but no similar spatial pattern in pathogen virulence, or correlation between mean resistance and mean virulence of sympatric plant–pathogen pairs such as that observed by Thrall and Burdon (2003), were documented.

Given the congruence between the cline in resistance structure and the pattern of field-measured infection prevalence, evidence of temporal stability in the prevalence cline, and high fitness costs of infection (Y. P. Springer, unpubl. ms.), it is unclear why disease does not drive hosts locally extinct in northern populations. Three general explanations are possible. First, the virulence structure of pathogen populations could be temporally variable. Given the annual bottlenecks and associated genetic drift characteristic of the lifecycle of *M. lini*, the relative abundance and diversity of rust pathotypes might fluctuate across years (Burdon 1993). Such fluctuations, which have been observed in other plant–rust interactions (Burdon and Jarosz 1992), could result in northern *H. californicum* populations being highly resistant to infection in certain years. Inoculation results provide mixed evidence for

this mechanism. On one hand, the majority of seeds used in this experiment were collected in 2002, the survey year in which the highest infection rates were observed in the field and in which many host populations, particularly those in the north, were decimated by disease. Presumably the plants that survived to seed set that year possessed high levels of genetic resistance to the rust pathotype(s) present at the time. These resistance genes proved of little benefit when plants were inoculated with rust samples collected in 2003 and 2004, suggesting rapid and dramatic changes in fungal virulence structure across years. In contrast, the fact that virulence patterns of the rust samples collected in 2003 were qualitatively similar to those collected in 2004, and that all 10 samples were consistently most virulent on hosts from northern populations, suggest that fungal pathotype diversity may be relatively stable. Second, rapid evolution of novel host resistance genotypes could allow host populations to quickly respond to pathogen attack (Thrall and Jarosz 1994; Duffy and Sivars-Becker 2007). Although *H. californicum* is an annual with a short generation time, this mechanism seems unlikely given that, in the face of high fitness costs of infection, exceptionally high rates of evolution would be required to produce this demographic rescue effect (Burdon 1994; Burdon and Thompson 1995). The failure to observe adaptive changes in host resistance structure following pathogen epidemics has been noted in studies of other host-pathogen interactions and attributed to alternative mechanisms including fitness trade-offs, linkage disequilibrium, weak frequency or density dependence due to poor pathogen transmission, and broadly virulent pathotypes capable of overcoming multiple host resistance genes (Parker 1991; Burdon and Thompson 1995; Little and Ebert 2001; Mitchell et al. 2004). Finally, spatiotemporal variation in abiotic conditions could confer environmental resistance on genetically susceptible host populations in some years. Quantification of temperature, relative humidity, and free-water availability over two years across the prevalence cline did not identify broad-scale latitudinal patterns in any of the three variables but did suggest considerable interannual consistency of conditions within sites (Y. P. Springer, unpubl. ms.). Inoculation experiments and field monitoring explicitly designed to examine the temporal dynamics of resistance and/or virulence structure and the abiotic conditions that might shape them are needed to rigorously assess these hypotheses.

This is one of a handful of studies that has investigated both disease pressure and resistance structure in a wild plant-pathogen interaction. The coincident clines in infection rates and genetic disease resistance suggest strong links between pathogen-mediated selection and host evolution and provide evidence of a clinal spatial pattern of pathogen-mediated evolution rarely documented in natural systems. The comprehensive biogeographic scale across which this pattern was characterized makes the results unique among studies of species interactions in natural ecosystems. By

moving beyond a focus on one or a few local interacting populations, such studies represent an important step toward understanding how host-pathogen interactions, and coevolution in general, create and maintain biodiversity across broad spatial scales.

#### ACKNOWLEDGMENTS

Thanks are due to P. Ainger, J. Callizo, C. Koehler, and S. Harrison for assistance in the field and to K. Andonian, L. Locatelli, D. Polk, and J. Velzy for greenhouse support. The manuscript was improved by comments from K. Andonian, M. Carr, G. Gilbert, J. Hoeksema, R. Kao, I. Parker, J. Shykoff, and three anonymous reviewers. Invaluable statistical guidance was provided by O. Kaltz and P. Raimondi. I also thank R. Calsbeek for encouragement, advice, and finger crossing. Financial support was provided by the Earl and Ethyl Meyers Oceanographic Trust, Friends of Long Marine Lab, and cofunding from the UCSC Department of Ecology and Evolutionary Biology and the Partnership for Interdisciplinary Studies of Coastal Oceans. Fieldwork was performed in part at the University of California natural reserve system McLaughlin Reserve.

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Associate Editor: J. Shykoff

## Supplementary Material

The following supplementary material is available for this article:

**Table A1.** Mean infection score and plant height for each plant population and mean virulence score for each fungal population. Infection score and virulence means calculated by pooling across all fungal samples and host populations, respectively. Plant infection score is a proxy for host susceptibility (inversely related to the level of genetic resistance). Letters indicate groupings produced by *t*-tests of all pairwise combinations of least-squares means adjusted using the Tukey HSD procedure (populations not connected by the same letter are significantly different [ $P < 0.05$ ]).

**Figure A1.** Mean infection score associated with each of the 10 fungal samples as a function of population latitude. Means calculated across all 16 plant populations. Error bars denote one standard error.

**Figure A2.** Relationship between mean resistance of host populations and mean virulence of associated fungal samples. Error bars denote one standard error. Subscripts identify source populations. Means are averages of greenhouse-measured infection scores calculated by pooling across all fungal samples or host populations, respectively.

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