

ECOLOGICAL GENETICS OF THE *BROMUS TECTORUM* (POACEAE)– *USTILAGO BULLATA* (USTILAGINACEAE) PATHOSYSTEM: A ROLE FOR FREQUENCY-DEPENDENT SELECTION?¹

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- **Premise of the study:** Evolutionary processes that maintain genetic diversity in plants are likely to include selection imposed by pathogens. Negative frequency-dependent selection is a mechanism for maintenance of resistance polymorphism in plant–pathogen interactions. We explored whether such selection operates in the *Bromus tectorum*–*Ustilago bullata* pathosystem. Gene-for-gene relationships between resistance and avirulence loci have been demonstrated for this pathosystem.
- **Methods:** We used molecular markers and cross-inoculation trials to learn whether the SSR genotypes of the host exhibited resistance to co-occurring pathogen races, whether host genotypes within a population had equal disease probability, and whether a common resistance locus and its corresponding avirulence locus exhibited predicted allele frequency changes during an epidemic.
- **Key results:** Five of six putative resistance loci that conferred resistance to co-occurring pathogen races occurred in common host SSR genotypes. Some common genotypes within populations were more likely to be diseased than others, and genotype frequencies sometimes changed across years in patterns consistent with frequency-dependent selection. Observed changes in frequency of resistance and virulence alleles during an epidemic provided further support, but evidence was inconclusive.
- **Conclusions:** Frequency-dependent selection may operate at endemic disease levels in this pathosystem, but is difficult to detect because many susceptible plants escape infection. Most pathogen isolates were virulent on most host genotypes, minimizing the apparent importance of frequency-dependent selection even during epidemics.

Key words: balanced polymorphism; *Bromus tectorum*; cheatgrass; frequency-dependent selection; gene-for-gene model; head smut disease; Poaceae; resistance; Ustilaginaceae; *Ustilago bullata*; virulence.

A fundamental question in plant biology is how plants and their pathogens are able to coexist. It is clearly disadvantageous for a pathogen to drive its host to extinction, but for individual pathogen strains, increased virulence usually results in higher fitness (reviewed by Sacristán and García-Arenál, 2008). From the plant perspective, resistance to pathogen attack is only under positive selection in the presence of the pathogen; it will be under negative selection in the absence of the pathogen if it incurs fitness costs (Burdon and Thrall, 2003). Plant host resistance to pathogens can take many forms, but probably the best-studied pathosystems are those that involve host resistance genes that confer resistance to specific pathogen races or virulence phenotypes (Crute et al., 1997). This pattern of host resistance and pathogen virulence is referred to as a gene-for-gene interaction. The allele conferring resistance at a particular locus is thought to enable recognition of the gene product (elicitor) of

a specific pathogen locus termed an avirulence locus because it is associated with a lack of virulence (Crute, 1998). If the pathogen possesses the virulent allele at that locus, it does not produce this elicitor and thus escapes detection by the corresponding resistance phenotype in the host. Resistance is conferred only if the host has the resistance allele that can recognize the gene product of the avirulent allele at a specific avirulence locus.

A population genetic corollary of the gene-for-gene model is the idea that polymorphism in both host resistance and pathogen virulence can be maintained through negative frequency-dependent selection (Jayakar, 1970; Leonard, 1997). In the simplest models of frequency-dependent selection in gene-for-gene systems, with a single resistance locus in the host and a single corresponding avirulence locus in the pathogen, the more frequent alleles in both host and pathogen are under negative frequency-dependent selection. This selection is indirect, because the selection pressure on a particular allele in the host is determined by the frequency of the corresponding avirulence allele in the pathogen, rather than on its own frequency. These models predict that an increase in the frequency of the allele conferring resistance at a resistance locus in a host population will select for a decrease in the frequency of the avirulent allele at the corresponding avirulence locus. The models propose a cost to maintaining both excess resistance and excess virulence, that should in turn over time result in a decrease in the frequency of the host resistance allele and a subsequent increase in the frequency of the pathogen avirulence allele. The net effect

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is that more frequent alleles are selected against, while less frequent alleles are favored, theoretically producing cyclic changes in allele frequencies at both host resistance and corresponding pathogen avirulence loci.

Fundamental to these models is the idea of fitness costs (Leonard, 1997). They propose that, if excess virulence had no cost, all alleles for virulence would become fixed in the pathogen population and host resistance would become selectively neutral. Similarly, if excess resistance had no cost, host populations could maintain high frequencies of an arsenal of resistance alleles regardless of whether they were immediately necessary. More recent models have shown that the patterns predicted by negative frequency-dependent selection described above can persist in the short term given only indirect selection (Tellier and Brown, 2007). Some form of direct selection is needed, however, for the system to attain balanced polymorphism over the long term, even if modest fitness costs for virulence and resistance are included. Because population dynamics of both host and pathogen involve many variables other than gene-for-gene interactions, the theoretical equilibrium state of balanced polymorphism is probably rarely if ever achieved in natural populations.

The idea that particular resistance phenotypes of plants and corresponding virulence genotypes of the pathogen can coexist has been termed the trench warfare model of host–pathogen interaction. An alternative idea is the arms race model, supported by observations in crop systems, where new resistance genes must be continually introduced into the crop as the pathogen evolves virulence that can overcome previously introduced resistance genes (Salvaudon et al., 2008). Whether the arms race model applies to natural pathosystems is not clear.

The models discussed have no spatial component; they assume large, panmictic populations with no provision for dispersal or gene flow. Most investigations of natural pathosystems have indicated that metapopulation dynamics are at least as important as population-level selection in mediating frequency changes in resistance and avirulence alleles (Thrall and Burdon, 1997). Some models indicate that the condition of fitness costs for maintenance of polymorphism is relaxed if metapopulation dynamics are introduced (Thrall and Burdon, 2002). It has not yet been demonstrated conclusively that resistance and virulence polymorphism are maintained by negative frequency-dependent selection in any natural pathosystem, although the work of Chaboudez and Burdon (1995) on the *Chondrilla juncea*–*Puccinia chondrillina* pathosystem offers empirical support for this theory. Dybdahl and Lively (1998) have demonstrated similar patterns of negative frequency-dependent selection for a snail–trematode system.

Studies have demonstrated that the spatial distribution of resistance phenotypes can mirror spatial variation in the probability of disease, with increased resistance in “evolutionary hotspots” where disease prevalence was high, both on a local scale (Laine, 2006) and across the range of a host species (Springer, 2007). Local adaptation has also been demonstrated for the pathogen in the *Linum marginale*–*Melampsora lini* pathosystem, manifested as lower levels of resistance to cooccurring pathogen populations than to non-cooccurring populations (Thrall et al., 2002). But in each host population, at least some fraction was resistant to cooccurring pathogen races, making it likely that frequency-dependent selection is part of the disease dynamic in these populations. In this pathosystem and others, comparisons of pre- and postepidemic host population structures have generally detected shifts in resistance allele

frequencies that appear to be maladaptive, i.e., not the result of selection as described in models of negative frequency dependence (e.g., Parker, 1991; Burdon and Thompson, 1995). Reciprocal selection on host and pathogen may also be delayed and thus be out of phase, making both local adaptation and frequency-dependent selection hard to detect, especially with a population sample from a single point in time (Dybdahl and Storer, 2003). In addition, when disease levels are endemic rather than epidemic, many susceptible individuals can escape infection, making selection weak and difficult to detect (Salvaudon et al., 2008).

Much of the research on both classical and population genetics of natural pathosystems has been with nonsystemic, biotrophic pathogens that have polycyclic life cycles (e.g., *Melampsora lini*: Burdon and Thompson, 1995; *Erysiphe fischeri*: Bevan et al., 1993; Laine, 2005). Burdon et al. (1996) predicted that race-specific resistance will be most common in pathosystems when the pathogen has a high probability of local extinction, i.e., when hosts or host tissues are annual and the pathogen is obligately biotrophic but not systemic. They have also proposed that gene-for-gene interactions should be more common in pathosystems in which the pathogen disperses farther than its host (Thrall and Burdon, 1997).

Smuts and bunts (Ustilaginales) are among the few systemic pathogens for which race-specific resistance has been demonstrated (Fischer and Holton, 1957; Thomas, 1991). The model of Tellier and Brown (2007) may explain why resistance polymorphism involving monocyclic systemic pathogens is uncommon. Their model indicates that a polycyclic pathogen, which can infect the same host individual multiple times, can exert selection pressure that results in direct negative frequency-dependent selection in the host, a condition for balanced polymorphism. Such selection pressure makes it more likely that polymorphism will be detected because it will tend to be more persistent. In our study, we examined evidence of negative frequency-dependent selection in a natural pathosystem involving the smut pathogen *Ustilago bullata* Berk. [syn. *U. bromivora* (Tul.) Fisch. von Waldh] and the invasive winter annual grass *Bromus tectorum* L.

Ustilago bullata, the causal organism of head smut of grasses, is a highly polymorphic species that can attack several genera of grass hosts (Fischer, 1940). It is a seedling-infecting pathogen that grows systemically in host vegetative tissues, then preempts flowering tissue for its own reproduction (Fig. 1). In most cases, particularly in annual hosts, successful infection by this pathogen completely prevents seed production. The life cycle of the pathogen in an annual plant is matched to that of its host, with a single generation per year (i.e., a monocyclic life cycle).

According to early studies, race-specific resistance at the species level is the norm for host response to *U. bullata* (Fischer, 1940; Meiners and Fischer, 1953). In addition, pathogen races defined on the basis of a series of host differential species were often complex genetic entities made up of multiple avirulence genotypes, with contrasting patterns of race-specific resistance on different populations within native grass species (Kreizinger et al., 1947).

Bromus tectorum is an important host for *U. bullata* in western North America (Fischer, 1940; Meyer et al., 2001). This highly inbreeding exotic winter annual grass has populations that can be characterized as assemblages of inbreeding lines with characteristic single sequence repeat (SSR or microsatellite) marker genotypes (Ramakrishnan et al., 2006). Four-locus

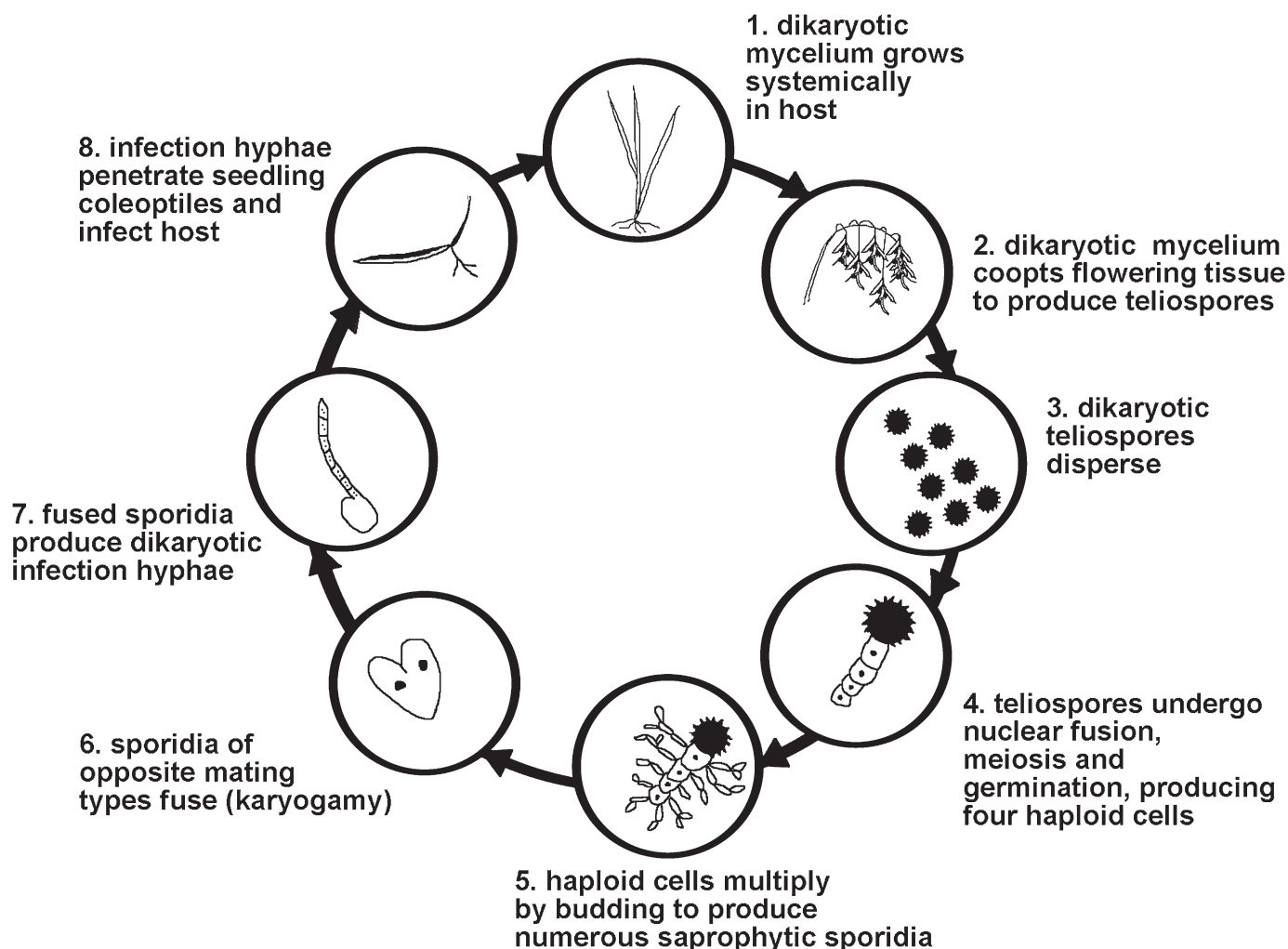


Fig. 1. Schematic representation of the life cycle of *Ustilago bullata* on *Bromus tectorum*.

SSR genotype is strongly correlated with several potentially adaptive traits in this species, including *U. bullata* resistance phenotype (Ramakrishnan et al., 2004). Although we have evidence from associated AFLP markers that members of a single SSR genotype are less closely related than members of a clone, they were shown to be more closely related to each other than to other SSR genotypes in the same population (Ramakrishnan et al., 2004). Levels of heterozygosity in this species average much less than 1%, and in a survey of almost a hundred populations regionwide, most populations had no detectable levels of heterozygosity (S. E. Meyer, unpublished data). These very high levels of inbreeding make it possible to use the SSR genotype as a proxy for other traits, including the head smut resistance phenotype. The correlation between SSR genotype and resistance phenotype is not perfect, as noted by Meyer et al. (2005). It is likely, however, that most if not all members of an SSR genotype share a close common ancestry, especially within a population, and are therefore much more likely to share a resistance phenotype than members of different SSR lineages.

We have demonstrated that the *Bromus tectorum*–*Ustilago bullata* pathosystem exhibits resistance/avirulence polymorphism that is consistent with the gene-for-gene model (Meyer

et al., 2001, 2005). These studies used small numbers of host lines and generally failed to detect host resistance against cooccurring pathogen races. Within-population host resistance polymorphism with corresponding virulence polymorphism in the cooccurring pathogen population is a necessary first condition for frequency-dependent selection (Crute et al., 1997). In the present investigation, we first asked whether such host resistance polymorphism was present in any of the populations we studied. Second, we asked whether different SSR genotypes within a population had equal disease probability, and whether disease probability for a particular SSR genotype changed across years, as might be expected if different host lines were subject to differential pathogen selection pressure as a function of the mix of pathogen races present. A nonrandom distribution of SSR genotypes in smutted vs. unsmutted categories could provide indirect evidence that corresponding resistance phenotype frequencies represent a response to selection by a specific array of pathogen races. Third, we attempted to test the model of frequency-dependent selection directly by measuring the change in the frequency of resistance at a common host resistance locus and in the frequency of virulence at the corresponding pathogen avirulence locus during the course of a severe head smut epidemic.

MATERIALS AND METHODS

Screening for host resistance and pathogen avirulence—To detect host resistance loci with corresponding avirulence loci in the cooccurring pathogen race, we selected lines with unique four-locus SSR genotypes from each of four intensively studied populations: Whiterocks, Hobbie Creek, and Strawberry, Utah, and Potosi Pass, Nevada (see Ramakrishnan et al., 2006 for complete lists of SSR genotypes and Meyer et al., 2005 for site information). We reasoned that systematically including all known SSR genotypes for each population should maximize the chances of encountering resistance genes. This selection method resulted in the inclusion of 21 host lines for Whiterocks, 14 host lines for Hobbie Creek, nine host lines for Strawberry, and two host lines for Potosi Pass. Host lines were inoculated only with a set of pathogen isolates from the cooccurring pathogen population. Original bulk inoculum and host line collections for these tests were made in 1998.

We used protocols described in Meyer et al. (2005) for the cross-inoculation trials. We used the same sets of pathogen isolates that were used in the earlier trials, which had been stored as sporidial cultures at -80°C to preserve viability. The exception was the Hobbie Creek population, for which the original isolates were lost to contamination, and new isolates had to be obtained. To obtain monosporidial lines, we atomized teliospores on potato dextrose agar with antibiotics (PDAA) for germination (Fig. 1). Resulting sporidia were transferred aseptically to sterile water, spread on the surface of water agar, and allowed to proliferate briefly. Using a tetrad microscope, we transferred individual sporidial colonies from water agar to potato dextrose broth for shaker culture at room temperature to produce monosporidial cultures. The cultures were tested for mating type by pairing in all combinations on PDAA and observing whether mating occurred, to ensure that both mating types were included in isolate pairs for inoculation. For each host line $\times$ pathogen isolate combination, 12 seeds (florets) of each host line were immersed in a mixture of two monosporidial pathogen isolates, one of each mating type. The seeds were then sown into containers, grown to the three-leaf stage, vernalized at $2\text{--}5^{\circ}\text{C}$ for 6–8 wk, and grown to flowering. Disease incidence was scored as the proportion of individual plants for each host line $\times$ paired monosporidial isolate combination that developed smutting. We included a total of 552 cross-inoculations and 6624 planted seeds.

Most often the distinction between susceptible and resistant host reactions was clear, with susceptible reactions giving 90–100% smutted plants, but some inoculations produced intermediate levels of smutting (Appendix S1, see Supplemental Data at <http://www.amjbot.org/cgi/content/full/ajb.0900261/DC1>). Only combinations that resulted in $<10\%$ smutted individuals were scored as indicating host resistance. We used patterns of response to the set of pathogen isolates for each population to identify putative resistance loci and to group host lines that had identical patterns of resistance. In our previous study with 13 host lines and 12 pairs of isolates from each of four pathogen populations, we had identified four putative resistance loci (Meyer et al., 2005). Subsequent work revealed that some of the corresponding pathogen avirulence phenotypes are controlled by single loci with simple Mendelian inheritance and follow the classical gene-for-gene model, while others have more complex patterns of genetic control (S. E. Meyer and S. Clement, unpublished data). Crossing experiments are required to distinguish among these modes of inheritance. For ease of presentation, we ascribe each unique pattern of resistance observed to a single resistance/avirulence locus, even though this undoubtedly represents an oversimplification in some cases. In the current study, we also examined patterns of resistance in response to sets of isolates common to the two cross-inoculation studies to determine whether new host lines showing resistance possessed resistance alleles identified in the earlier study. We included two host lines known to have resistance to pathogen races from the previous test in the Hobbie Creek cross-inoculations, to determine whether any of the newly obtained isolates represented known pathogen races.

Because the SSR genotypes of the host lines used in this study and their frequencies in the host populations were known, we were able to determine whether particular patterns of resistance occurred in SSR genotypes that were common or rare in a particular population. Common SSR genotypes for each population were defined as those occurring at $>10\%$ frequency in at least one subpopulation and year (Ramakrishnan et al., 2006). The occurrence of a resistance phenotype in a common SSR genotype does not prove that the resistance phenotype is also common, but it does increase the probability that this is the case. Similarly, if a resistance phenotype is found only in a rare SSR genotype, it too is more likely to be rare.

Detecting differences in SSR genotype frequency as a function of disease—We examined the relative frequency of common SSR genotypes in the smutted and unsmutted subsets of populations at Whiterocks and Hobbie Creek in 1999 and 2000. The protocol for DNA extraction from field-collected tissue

for this study is described in Ramakrishnan et al. (2006). The SSR genotype frequency data presented in this earlier paper for 1999 and 2000 for these two populations are based on the same data set analyzed here. In the earlier paper, we used these data to characterize changes in frequencies of different SSR genotypes across years, without regard to whether the individual genotyped was smutted or unsmutted. In the present investigation, the distribution of these genotypes within unsmutted and smutted categories was our focus. We collected 100 individuals each in the smutted and unsmutted categories from each population in 1999 and 50 individuals in each category in 2000. Because of problems extracting high-quality DNA from already-senescent leaf tissue of plants mature enough to score as smutted or unsmutted, the usable sample size was sometimes smaller.

We used contingency table analysis based on count data to test for differences in the frequency distribution of common genotypes within the smutted vs. unsmutted category for each population and year. Rare genotypes were excluded to meet requirements for χ^2 analysis; reported sample sizes exclude individuals with rare genotypes (<5 individuals per disease category).

We also measured disease incidence in each host population in 1999 and 2000. Relative density of smutted individuals was obtained from a random sample of ten 0.093-m^2 plots in each population each year. Within each plot, all individuals were counted and scored as smutted or unsmutted, and the proportion of smutted plants was calculated. These proportions were averaged to obtain mean disease incidence.

Measuring changes in frequency of resistance and avirulence during an epidemic—To test the idea that frequency-dependent selection would be more easily detected under an epidemic scenario, we chose a study site near Arrowrock Reservoir in the foothills above Boise, Idaho, where head smut epidemics had been previously reported. This population occurred as a near-monoculture over several hectares. In 1999, we observed relatively high levels of smutting (estimated at 50%) in this population. We collected seeds of 100 randomly selected unsmutted individuals, and obtained a representative bulk inoculum collection by harvesting smutted heads from at least 100 randomly selected individuals. We returned in 2001 and resampled the population in the same way. At that point, we observed very high levels of smutting (estimated at $>95\%$). Finally, we returned in 2003. There were very few *B. tectorum* plants in evidence. Most of the site was dominated by *Poa bulbosa*, a species that can persist with *B. tectorum* as a suppressed understory. It often quickly rebounds in abundance after *B. tectorum* removal, for example, following herbicide use (S. E. Meyer, personal observation). We were able to locate 19 host plants, none of which were smutted, and we collected seeds of these individuals.

In autumn 2001, we inoculated seeds of 90 host lines collected in 1999 and in 2001 with 1999 and 2001 bulk inoculum, using a protocol similar to that described for resistance screening, except that the seeds were placed in vials and inoculated with dry teliospores by vibration (Meyer et al., 2001). From this test, we identified eight host lines from each collection year that had relatively low levels of disease after bulk inoculation. We used these host lines to screen paired sporidial isolates obtained at random from the bulk inoculum collected each year, on the logic that these host lines would be more likely to have resistance genes that would permit us to detect avirulence loci in the pathogen population. We inoculated these 16 host lines with 30 paired isolates from the 1999 bulk inoculum and 30 paired isolates from the 2001 bulk inoculum. Because of space limitations, this cross-inoculation trial took place in two installments with initiation dates in fall 2002 and fall 2003. Each installment included equal numbers of isolates from each collection year. As there was no significant difference between trial years, these data were pooled for analysis. The protocol was similar to that used in the screening trials described earlier, with 12 seeds per host line by paired isolate combination for a total of 480 inoculations and 5760 planted seeds. We looked for common patterns of disease susceptibility and resistance to identify putative resistance loci and their corresponding avirulence loci. We then quantified the frequency of avirulent and virulent alleles at each putative avirulence locus for each year of collection.

Once we had identified the most common avirulence phenotype in the pathogen population, we screened all available host lines from each year of collection for resistance to this pathogen race; these host lines were first increased in the greenhouse to provide sufficient seeds for the inoculation trials (Meyer et al., 2001). We used six paired isolates of known virulence phenotype (i.e., three virulent and three avirulent on the original set of resistant host tester lines) to quantify the frequency of the corresponding resistance phenotype in the host population each year. These six paired isolates were used to inoculate 56 additional host lines from 1999 and 56 additional host lines from 2001. We also included 19 host lines collected in 2003, resulting in a total of 786 cross-inoculations and 9432 planted seeds. This cross-inoculation trial was also carried

out in two installments, with equal representation of collection years in each installment, and initiation dates in fall 2004 and fall 2005. There were no significant differences between trial years, and data were again pooled for analysis.

RESULTS

Screening for host resistance and pathogen avirulence—Screening for host resistance loci complementary to avirulence loci in the corresponding pathogen population for four host populations resulted in the discovery of four new putative resistance loci (Table 1). At Whiterocks, 15 of 21 lines were susceptible to all 12 isolate pairs (see online Appendix S1 for detailed results of cross-inoculations for each population). One line showed a pattern of resistance indicating that it possessed UB₂, a resistance allele detected earlier in the Hobbie Creek population but not in the Whiterocks population (Meyer et al., 2005). The corresponding avirulence locus, avr_{UB2}, had been reported from the Whiterocks and Strawberry pathogen populations. Two additional putative resistance loci (UB₅ and UB₆) were detected in the Whiterocks host population. Because only 12 isolate pairs were used, frequencies must be interpreted with caution, but the frequency of avirulence varied from 0.08 for avr_{UB5} and avr_{UB6} to 0.25 for avr_{UB2}. The SSR genotypes that possessed resistance belonged to both common and rare subsets of the Whiterocks population, but each resistance phenotype was found in at least one common SSR genotype as defined in Ramakrishnan et al. (2006).

For Hobbie Creek, eight of 14 SSR genotypes were susceptible to all 10 isolate pairs (Table 1). One of the lines demonstrated to have UB₂ in the earlier test, Hobbie Creek line HC02, was also resistant to two of the isolate pairs in the present test. The most parsimonious explanation is that these two isolate

pairs also possess avr_{UB2}. Two common SSR genotypes at Hobbie Creek exhibited evidence of a new resistance locus (UB₇) that corresponded to an avirulence locus (avr_{UB7}) with avirulence at high frequency. Three rare host genotypes possessed a resistance phenotype indicating the presence of a second new resistance gene in the Hobbie Creek host population, UB₈. This resistance gene rendered these lines resistant to all 10 pathogen isolate pairs used in the trial, which all therefore possessed avr_{UB8}. In terms of the Hobbie Creek pathogen isolates tested, these three host genotypes were universally resistant.

At Strawberry, no new resistance genes were identified, but UB₂, previously known only from the Hobbie Creek population (and also detected in the current trials in the Whiterocks population), was detected in two host lines (Table 1).

The Potosi Pass host population consists of two SSR genotypes, one of which is very common and one of which is uncommon (Ramakrishnan et al., 2006). In our earlier tests, we demonstrated that the common Potosi Pass SSR genotype was resistant, not only to all known pathogen races from the three northern populations, but also to some isolates from the co-occurring pathogen population (Meyer et al., 2001, 2005). We designated its putative resistance gene as UB₁. In the inoculation trials performed for the current research, we confirmed that the less common SSR genotype was susceptible to all Potosi Pass pathogen isolates, whereas the common genotype was susceptible only to isolates virulent at the avr_{UB1} locus.

We detected resistance phenotypes in all four host populations that corresponded to putative avr genes in co-occurring pathogen populations, meeting the first condition for the operation of frequency-dependent selection. Most resistance phenotypes were detected in common SSR genotypes, making it more likely that they play a significant role in host–pathogen population dynamics. However, most host SSR genotypes (31 of 46

TABLE 1. Putative resistance and avirulence genes identified in cooccurring host and pathogen populations in inoculation trials with all known SSR genotypes from each of four *Bromus tectorum* populations and sets of 10–12 paired monosporial isolates from cooccurring pathogen populations used in previous work (Meyer et al., 2005). See online Appendix S1 for complete results.

Population	R gene ^a	Host lines	Host line SSR genotype	avr gene ^a	Pathogen isolate pairs ^b	Avirulence frequency
Whiterocks (15 of 21 lines universally susceptible)	UB ₂	15-19	JCBB ^c	avr _{UB2}	WR151, WR157, WR284	0.25
	UB ₅	15-15	DCBB ^c	avr _{UB5}	WR281	0.08
		15-69	ECBB			
		15-70	GCAB			
	UB ₆	15-18	IEBB ^c	avr _{UB6}	WR183	0.08
		15-44	CCBB			
Hobbie Creek (8 of 14 lines universally susceptible)	UB ₂	18-2	GCBB ^c	avr _{UB2}	HC151n, HC184n	0.20
	UB ₇	18-50	GCBB ^c	avr _{UB7}	HC151n, HC158n, HC1510n, HC183n,	0.80
		18-60	DCBB ^c		HC184n, HC185n, HC281n, HC284n	
	UB ₈	18-17	ECBF	avr _{UB8}	All isolate pairs had the avirulent form of the avr ₈ gene	1.00
		18-107	CCBB			
		18-108	ECCA			
Strawberry (7 of 9 lines universally susceptible)	UB ₂	28-65	JABB	avr _{UB2}	ST154, ST1510, ST185	0.33
		28-92	DABB ^c			
Potosi Pass (1 of 2 lines universally susceptible)	UB ₁	20-1	FEDD ^c	avr _{UB1}	PP154, PP158, PP182, PP185, PP281, PP2842	0.46 ^d

^a R and avr numbers in Meyer et al. (2005) have been replaced with more specific designators.

^b Isolate labels as in Meyer et al. (2005), except for new HC isolates, designated with the letter n.

^c SSR genotypes designated as common in Ramakrishnan et al. (2006).

^d Data from Meyer et al. (2005).

tested) had no detectable resistance to cooccurring pathogen races, and many pathogen isolates were virulent on most or all genotypes in cooccurring host populations.

Detecting differences in SSR genotype frequency as a function of disease—Common SSR genotypes were not distributed randomly with respect to the smutted and unsmutted subsets of the population at either Hobble Creek or Whiterocks. At Hobble Creek, disease incidence increased substantially from 1999 (34%) to 2000 (64%). In 1999, the frequency distribution for the four common genotypes did not differ significantly in smutted vs. unsmutted subsets of the population (Fig. 2A). But in 2000, this difference was highly significant (Fig. 2B). The most common genotype, DCBB, represented about 40% of the total plants each year, but in 1999 these plants were distributed about equally in the smutted and unsmutted categories, whereas in 2000 this genotype was massively overrepresented in the smutted category. This is clearly a case where the most frequent genotype experienced negative selection imposed by the pathogen. The second most common genotype, JCBB, showed the opposite response, essentially disappearing from the smutted category in 2000. These results suggest that the selection pressure applied by the increase in disease incidence affected different SSR genotypes differentially. This may have been due to the relationship between their resistance phenotypes and the frequency of different virulence phenotypes in the pathogen population.

At Whiterocks, disease incidence was present at endemic levels in both 1999 (28%) and 2000 (33%). In 1999, the frequency distribution for eight common genotypes differed significantly in smutted vs. unsmutted subsets of the population. The most common genotype, DCBB, was underrepresented in the smutted category, whereas the three next most common genotypes were overrepresented (Fig. 2C). A very similar pattern was seen in 2000, though in this case the difference was only marginally significant, probably because of the smaller sample size (Fig. 2D). The genotype DCBB apparently increased in total frequency at the expense of less common genotypes, but the basic pattern remained unchanged. These patterns suggest that selection, possibly based on race composition of the pathogen population, operates differentially on different genotypes when disease levels are endemic, but that shifts in relative abundance of genotypes may take longer to evidence themselves.

Measuring changes in frequency of resistance and avirulence during an epidemic—We detected five resistance phenotypes in the host population at Arrowrock, Idaho. In the cross-inoculation trials with 16 host tester lines and 60 isolate pairs, one host line in the 2001 collection was resistant to all 60 paired pathogen isolates; it also showed disease incidence of <10% in bulk inoculation trials with both 1999 and 2001 bulk inoculum, suggesting that it possesses a resistance phenotype that renders it resistant to essentially all races in the cooccurring pathogen population. One host line in the 1999 collection was resistant to a single isolate pair, indicating the existence of a unique resistance phenotype. Seven of 16 host tester lines showed an identical pattern of resistance, with resistance to 31 of the 60 paired isolates. The high frequency of both resistance and avirulence indicated that this putative gene-for-gene interaction could potentially be important in disease dynamics, and it became the focus of our further study. In the cross-inoculation of 131 additional host lines with six isolate pairs of known viru-

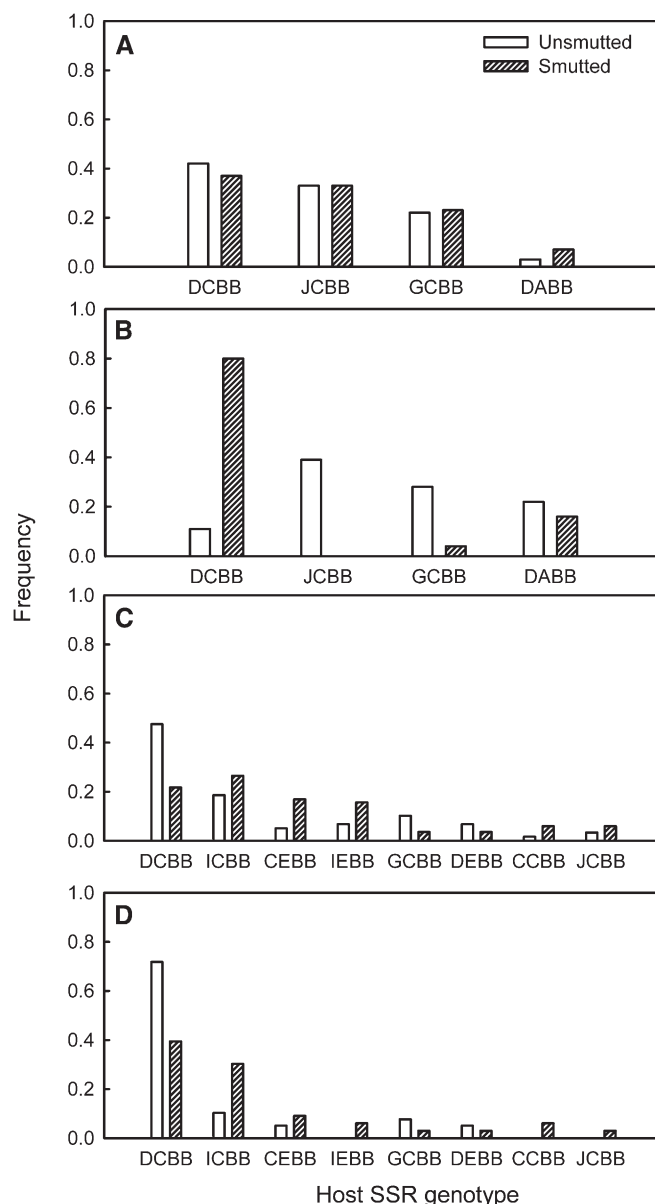


Fig. 2. Frequency of common genotypes ($N > 5$ in at least one subset) in smutted and unsmutted subsets of the population at (A) Hobble Creek in 1999 ($\chi^2 = 1.43$, $N = 133$, $df = 3$, $P = 0.697$), (B) Hobble Creek in 2000 ($\chi^2 = 21.6$, $N = 43$, $df = 3$, $P < 0.001$), (C) Whiterocks in 1999 ($\chi^2 = 19.3$, $N = 142$, $df = 7$, $P = 0.007$), and (D) Whiterocks in 2000 ($\chi^2 = 14.2$, $N = 72$, $df = 7$, $P = 0.048$).

lence phenotype, we detected only two additional resistance phenotypes, both rare (two host lines for each resistance phenotype). Most host lines at Arrowrock (77%) were susceptible to all 60 pathogen isolate pairs.

Contingency table analysis revealed that the relative frequency of avirulent isolate pairs for the common avirulence locus decreased significantly over the course of the epidemic, dropping from 77% in 1999 to 27% in 2001 ($\chi^2 = 15.0$, $N = 60$, $df = 1$, $P = 0.0003$; Fig. 3A). We did not see a corresponding increase in the frequency of host lines resistant to this pathogen race over the same period (17% in 1999 vs. 16% in 2001; Fig. 3B). However, the frequency of resistance in the 2003

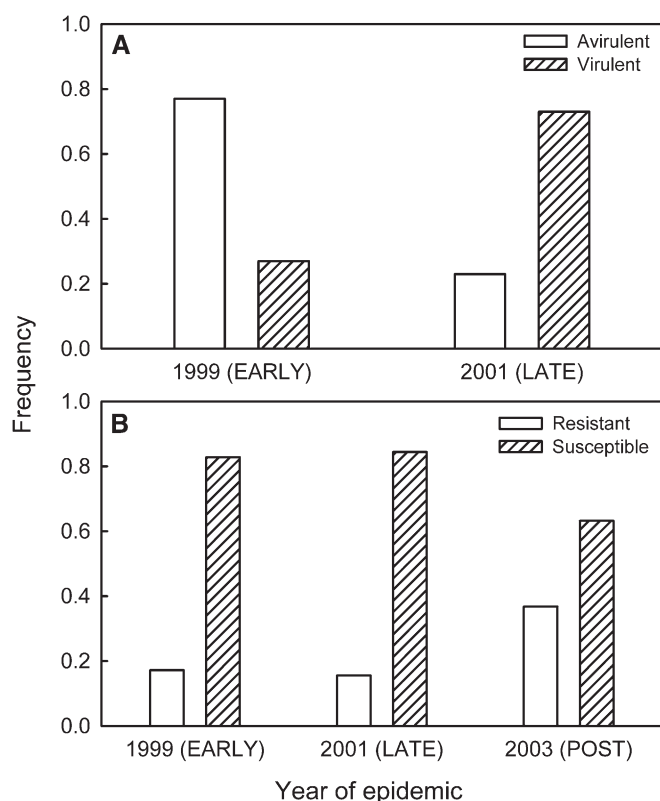


Fig. 3. Changes in the frequency of (A) virulent and avirulent isolates carrying a common avirulence gene and (B) of resistant and susceptible individuals carrying the corresponding resistance gene, during a severe head smut epidemic on *Bromus tectorum* at Arrowrock, Idaho. Early and late sampling dates are significantly different for relative frequencies of virulent and avirulent pathogen isolates ($\chi^2 = 15.0$, $N = 60$, $df = 1$, $P = 0.0003$). Postepidemic sampling date is significantly different from early and late sampling dates for relative frequencies of resistant and susceptible host lines ($\chi^2 = 4.48$, $N = 131$, $df = 1$, $P = 0.034$).

postepidemic population sample was over twice as high as in the population samples taken previously, a significant increase (37%; $\chi^2 = 4.48$, $N = 147$, $df = 1$, $P = 0.034$). The significant increase we saw in the frequency of the virulent pathogen race was therefore accompanied by a significant increase in the frequency of the resistant host phenotype. These shifts were in the direction predicted by negative frequency-dependent selection. In both host and pathogen, the common phenotype apparently underwent negative selection. However, the shifts in frequency were out of phase. The measured decrease in the frequency of the avirulent pathogen race apparently took place before the measured increase in frequency of host resistance, rather than in response to it.

DISCUSSION

We used three studies to address the role frequency-dependent selection as a mechanism for the maintenance of resistance polymorphism in the *B. tectorum*–*U. bullata* pathosystem. First, in screening all SSR genotypes in each of four host populations, we found at least one putative resistance locus in each host population that corresponded to an avirulence locus in the cooccurring pathogen population (Table 1). Five of six loci confer-

ring resistance to cooccurring pathogen races were found in common SSR genotypes and are therefore likely to be common. Detectable avirulent alleles at corresponding avirulence loci were also relatively common in cooccurring pathogen populations, suggesting that resistance could function to lower disease levels within cooccurring populations of host and pathogen and could provide the substrate for frequency-dependent selection. We included relatively few pathogen isolates from each population in our inoculation trials; it is possible that many avirulence genes and their corresponding resistance genes remain undetected. However, even with a much larger number of isolate pairs and host lines in the Arrowrock cross-inoculations, only five resistance loci were detected, so it is likely that the number of resistance loci is not greatly underestimated in tests with Utah and Nevada populations. It may be that the loci at Arrowrock correspond to those identified in other populations. Inoculation of lines with known resistance phenotypes from these other populations would be required to determine this. The number of resistance loci we identified is similar to that found in other natural pathosystems with race-specific resistance (Dinoor, 1977; Jarosz and Burdon, 1991; Parker, 1991).

The existence of apparently universally resistant host lines in both the Hobbie Creek and Arrowrock populations raised some interesting questions. We thought that perhaps the resistance expressed in these host lines might correspond to the UB_1 gene in the Potosi Pass population, which renders host individuals resistant to all races in the Whiterocks, Hobbie Creek, and Strawberry pathogen populations. But inoculating with teliospores from the Potosi Pass pathogen race that carries the virulent form of avr_{UB_1} yielded a resistant reaction for each of these universally resistant lines, demonstrating that their resistance genes do not correspond to the UB_1 locus in the Potosi Pass population (S. E. Meyer and S. Clement, unpublished data). The lines possessing these exceptional resistance genes seem to be consistently rare in their host populations, even under the scenario of epidemic levels of disease, suggesting that universal resistance must have a high fitness cost (Burdon and Thrall, 2003) or be otherwise associated with maladaptive traits in the inbreeding lines that exhibit it. Otherwise, we would expect such host lines to be strongly favored under epidemic conditions, and this was not the case for the single universally resistant line found at Arrowrock.

A second line of evidence that provided some support for frequency-dependent selection in this pathosystem was obtained from SSR genotype frequency distributions in smutted and unsmutted subsets of two populations. At Hobbie Creek, under conditions of increasing disease pressure, frequency distributions in smutted vs. unsmutted subsets changed from closely similar in 1999 to highly significantly different in 2000 (Fig. 1A, B). It seems plausible that the sharp increase in the proportion of common genotype DCBB in the smutted category could be related to an increase in frequency of a pathogen race that is virulent on this line. If the virulent form of the pathogen race that possessed the corresponding avr gene increased in frequency, a decrease in the frequency of unsmutted plants and an increase in the frequency of smutted plants of this SSR genotype would be the expected outcome. The accompanying decrease in seed production by this SSR genotype in 2000 would very likely result in a decrease in its absolute abundance in the next generation. Genotype DCBB at Hobbie Creek was found to carry the UB_7 gene, with the avirulent form of the corresponding avr_{UB_7} gene at apparently high frequency in the 1998 bulk pathogen inoculum collection (Table 1).

At Whiterocks, the significant difference in genotype frequency distributions between smutted and unsmutted categories was consistent across years (Fig. 1C, D). The DCBB genotype at Whiterocks was consistently less frequent in the smutted category than in the unsmutted category, while the three next most common genotypes were consistently more frequent in the smutted category. This could suggest that the DCBB genotype was resistant to a common pathogen race to which other common lines were susceptible. Genotype DCBB at Whiterocks was found to carry the *UB₅* gene. Because disease incidence was at endemic levels in this population across both years, we do not expect to see the sharp changes in allele frequencies that might be observed under epidemic conditions. The difference in the rate of change in frequency distributions, namely, rapid at Hobble Creek and slow at Whiterocks, is likely to be due to different intensities of selection for resistance that accompany different levels of disease.

It is not possible to tell from our data whether the DCBB lines in the two host populations had similar or different resistance phenotypes, because a resistance gene can be detected only if the host is challenged with a pathogen race that possesses the corresponding avirulence gene. We found *avr_{UB5}* only at Whiterocks and *avr_{UB7}* only at Hobble Creek, so it is possible that the DCBB lines in both populations possessed both resistance genes.

A third line of evidence addressing the maintenance of resistance polymorphism through frequency-dependent selection in this pathosystem was obtained by directly measuring changes in the frequency of virulent and avirulent isolates carrying a common avirulence locus and accompanying changes in the frequency of resistant and susceptible individuals carrying the corresponding resistance locus. We saw a clear decrease in the frequency of the avirulent form of the pathogen, which was at high frequency at the beginning of the study, over a 2-yr period during a severe epidemic. This suggests negative frequency-dependent selection, because the common virulence phenotype decreased significantly in abundance, while the rare phenotype increased. We also detected an increase in the frequency of host individuals carrying the corresponding resistance gene, which was at relatively low frequency at the beginning of the study. These patterns of change are superficially consistent with a frequency-dependent selection model, but closer examination showed that the changes in frequencies that should have been complementary were actually out of phase, so that the increase in frequency of resistance could not be directly invoked to explain the decrease in frequency of avirulence at the corresponding *avr* locus. The increase in the frequency of the resistant host genotype during the epidemic does represent an adaptive shift, however, in contrast to apparently maladaptive shifts observed in some other pathosystems (Parker, 1991; Burdon and Thompson, 1995).

It is possible that the failure to detect a change in resistance frequency over the first 2 years of the study was due to inadequate sample size. Unfortunately with a systemic castrating pathogen like *U. bullata*, where rearing plants to flowering is necessary for accurate disease diagnosis, truly large-scale experiments rapidly become prohibitively space and labor-intensive. Our efforts to shorten the experimental cycle by using PCR-based pathogen detection methods on vegetative host tissue were not successful, because some resistance phenotypes contain the pathogen in vegetative tissue even though they do not develop disease (A. Ramakrishnan unpublished data).

Our study demonstrates the potential for negative frequency-dependent selection in wild populations of *B. tectorum* and their cooccurring *U. bullata* populations. It also presents some indi-

rect evidence that the relative abundance of specific SSR genotypes in a given year might be related to their resistance phenotypes. But many of the SSR genotypes in this study had no detectable resistance genes effective against cooccurring pathogen races, while many pathogen isolates were virulent on almost all host lines, regardless of resistance phenotype. Local extinction events such as the one observed at the Arrowrock site are probably mediated more by environmental factors than by patterns of pathogen virulence and host resistance. This pathogen infects seedlings most effectively when germination-triggering rainfall events occur early in the fall, when temperatures are still moderate; cohorts that emerge later have a high probability of escape regardless of resistance phenotype (Boguena et al., 2007). When disease is at endemic levels due to environmental constraints, many susceptible host individuals escape infection, and the effect of host resistance phenotype may be difficult to detect (Salvaudon et al., 2008). Under epidemic conditions, few susceptible plants escape, and resistance would be expected to play a more important role. In the case of the epidemic we studied, however, the short-term result was local extinction of the pathogen and near-extinction of the host. This indicates that the probability of infection was so high under prevailing environmental conditions that any ameliorating short-term effect of selection for increased resistance was unable to manifest itself. Even though we were unable to demonstrate conclusively that frequency dependent selection is an important evolutionary force in this pathosystem, we provided some evidence that such selection could be occurring. The relative importance of selection on resistance phenotype vs. metapopulation processes such as dispersal and genetic drift in shaping population resistance structure in this pathosystem remains to be fully elucidated.

LITERATURE CITED

- BEVAN, J. R., I. R. CRUTE, AND D. D. CLARKE. 1993. Variation for virulence in *Erysiphe fischeri* from *Senecio vulgaris*. *Plant Pathology* 42: 622–635.
- BOGUENA, T., S. E. MEYER, AND D. L. NELSON. 2007. Low temperature during infection limits *Ustilago bullata* disease incidence on *Bromus tectorum*. *Biocontrol Science and Technology* 17: 33–52.
- BURDON, J. J., AND J. N. THOMPSON. 1995. Changed patterns of resistance in a population of *Linum marginale* attacked by the rust pathogen *Melampsora lini*. *Journal of Ecology* 83: 199–206.
- BURDON, J. J., AND P. H. THRALL. 2003. The fitness cost to plants of resistance to pathogens. *Genome Biology* 4: 227.
- BURDON, J. J., A. WENNSTROM, T. ELMQVIST, L. ERICSON, AND G. C. KIRBY. 1996. The role of race-specific resistance in natural plant populations. *Oikos* 76: 411–416.
- CHABOUDEZ, P., AND J. J. BURDON. 1995. Frequency-dependent selection in a wild plant–pathogen system. *Oecologia* 102: 490–493.
- CRUTE, I. R. 1998. The elucidation and exploitation of gene-for-gene recognition. *Plant Pathology* 47: 107–113.
- CRUTE, I. R., E. B. HOLUB, AND J. J. BURDON [eds.]. 1997. The gene-for-gene relationship in plant–parasite interactions. CAB International, Oxon, UK.
- DINOOR, A. 1977. Oat crown rust resistance in Israel. *Annals of the New York Academy of Sciences* 287: 357–366.
- DYBDAHL, M. F., AND C. M. LIVELY. 1998. Host–parasite coevolution: Evidence for rare advantage and time-lagged selection in a natural population. *Evolution* 52: 1057–1066.
- DYBDAHL, M. F., AND A. STORFER. 2003. Parasite local adaptation: Red queen versus suicide king. *Trends in Ecology & Evolution* 18: 523–530.
- FISCHER, G. W. 1940. Host specialization in the head smut of grasses, *Ustilago bullata*. *Phytopathology* 30: 991–1017.

- FISCHER, G. W., AND C. S. HOLTON. 1957. Biology and control of the smut fungi. Ronald Press, New York, New York, USA.
- JAROSZ, A. M., AND J. J. BURDON. 1991. Host-pathogen interactions in natural populations of *Linum marginale* and *Melampsora lini*. II. Local and regional variation in patterns of resistance and racial structure. *Evolution* 45: 1618–1627.
- JAYAKAR, S. D. 1970. A mathematical model for interaction of gene frequencies in a parasite and its host. *Theoretical Population Biology* 1: 140–164.
- KREIZINGER, E. J., G. W. FISCHER, AND A. G. LAW. 1947. Reactions of mountain brome and Canada wild-rye strains to head smut (*Ustilago bullata*). *Journal of Agricultural Research* 75: 105–111.
- LAINE, A. L. 2005. Spatial scale of local adaptation in a plant-pathogen metapopulation. *Journal of Evolutionary Biology* 18: 930–938.
- LAINE, A. L. 2006. Evolution of host resistance: looking for coevolutionary hotspots at small spatial scales. *Proceedings of the Royal Society of London, B, Biological Sciences* 273: 267–273.
- LEONARD, K. J. 1997. Modeling gene frequency dynamics. In I. R. Crute, E. B. Holub, and J. J. Burdon [eds.], The gene-for-gene relationship in plant-parasite interactions, 211–230. CAB International, Oxon, UK.
- MEINERS, J. P., AND G. W. FISCHER. 1953. Further studies on host specialization in the head smut of grasses, *Ustilago bullata*. *Phytopathology* 43: 200–203.
- MEYER, S. E., D. L. NELSON, AND S. CLEMENT. 2001. Evidence for resistance polymorphism in the *Bromus tectorum*–*Ustilago bullata* pathosystem: Implications for biocontrol. *Canadian Journal of Plant Pathology* 23: 19–27.
- MEYER, S. E., D. L. NELSON, J. WATERS, S. CLEMENT, M. STEVENS, AND D. J. FAIRBANKS. 2005. Genetic variation in *Ustilago bullata*: Molecular genetic markers and virulence on *Bromus tectorum* host lines. *International Journal of Plant Sciences* 166: 105–115.
- PARKER, M. A. 1991. Nonadaptive evolution of disease resistance in an annual legume. *Evolution* 45: 1209–1217.
- RAMAKRISHNAN, A. P., S. E. MEYER, C. COLEMAN, AND D. J. FAIRBANKS. 2006. Ecological significance of microsatellite variation in western North American populations of *Bromus tectorum*. *Plant Species Biology* 21: 61–73.
- RAMAKRISHNAN, A. P., S. E. MEYER, J. WATERS, C. COLEMAN, M. STEVENS, AND D. J. FAIRBANKS. 2004. Correlation between molecular genetic markers and adaptively significant variation in *Bromus tectorum* (Poaceae), an inbreeding annual grass. *American Journal of Botany* 91: 797–803.
- SACRISTÁN, S., AND F. GARCÍA-ARENÁL. 2008. The evolution of virulence and pathogenicity in plant pathogen populations. *Molecular Plant Biology* 9: 369–384.
- SALVAUDON, L., T. GIRAUD, AND J. SHYKOFF. 2008. Genetic diversity in natural populations: A fundamental component of plant-microbe interactions. *Current Opinion in Plant Biology* 11: 135–143.
- SPRINGER, Y. P. 2007. Clinal resistance structure and pathogen local adaptation in a serpentine flax-flax rust interaction. *Evolution* 61: 1812–1822.
- TELLIER, A., AND J. K. M. BROWN. 2007. Stability of genetic polymorphism in host-parasite interactions. *Proceedings of the Royal Society of London, B, Biological Science* 274: 809–817.
- THOMAS, P. L. 1991. Genetics of small grain smuts. *Annual Review of Phytopathology* 29: 137–148.
- THRALL, P. H., AND J. J. BURDON. 1997. Host-pathogen dynamics in a metapopulation context: The ecological and evolutionary consequences of being spatial. *Journal of Ecology* 85: 743–753.
- THRALL, P. H., AND J. J. BURDON. 2002. Evolution of gene-for-gene systems in metapopulations: The effect of spatial scale of host and pathogen dispersal. *Plant Pathology* 51: 169–184.
- THRALL, P. H., J. J. BURDON, AND J. D. BEVER. 2002. Local adaptation in the *Linum marginale*–*Melampsora lini* host-pathogen interaction. *Evolution* 56: 1340–1351.