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Rapid genetic change underpins antagonistic coevolution in a natural host-pathogen metapopulation

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Abstract

Antagonistic coevolution is a critical force driving the evolution of diversity, yet the selective processes underpinning reciprocal adaptive changes in nature are not well understood. Local adaptation studies demonstrate partner impacts on fitness and adaptive change, but do not directly expose genetic processes predicted by theory. Specifically, we have little knowledge of the relative importance of fluctuating selection vs. arms-race dynamics in maintaining polymorphism in natural systems where metapopulation processes predominate. We conducted cross-year epidemiological, infection and genetic studies of multiple wild host and pathogen populations in the *Linum-Melampsora* association. We observed asynchronous phenotypic fluctuations in resistance and infectivity among demes. Importantly, changes in allelic frequencies at pathogen infectivity loci, and in host recognition of these genetic variants, correlated with disease prevalence during natural epidemics. These data strongly support reciprocal coevolution maintaining balanced resistance and infectivity polymorphisms, and highlight the importance of characterising spatial and temporal dynamics in antagonistic interactions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary graphs and tables which provide further data to support the studies described in the main text

Figure S1 Map showing location of host and pathogen populations used in the cross-year *Linum-Melampsora* study

Figure S2 Correlation between allelic variants at the *AvrP123* and *AvrP4* loci which suggests these could be treated as haplotypes

Figure S3 Relationship between pathogen infectivity and the frequency of Avr allelic variants when combined as haplotypes

Table S1 Disease levels during epidemics in the natural *L. marginale* populations from which *M. lini* isolates were collected for the time-shift inoculation studies.

Table S2 Results of a generalized linear mixed model (GLMM) for infection data from the glasshouse inoculation trials.

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Keywords

disease; resistance; infectivity; gene-for-gene; epidemiology; negative frequency-dependence; selective sweeps; avirulence; pathogen effectors; Red Queen

INTRODUCTION

Genetic variation in host resistance mechanisms and pathogen infection strategies has long been accepted as a crucial factor influencing disease epidemiology in human, plant and animal systems (Bergelson *et al*. 2001; Lockett *et al*. 2001; Lively 2010). There is evidence of genetic variation in host resistance and pathogen infectivity in most natural systems that have been examined (Paterson *et al*. 1998; Thrall *et al*. 2001; Altermatt & Ebert 2008), yet few studies have linked such variation to adaptive change (Thrall *et al*. 2002; Lively *et al*. 2004; Laine 2006; Decaestecker *et al*. 2007). In particular, we have little knowledge of how variation at the molecular level influences the population and genetic dynamics of disease in nature (Stahl *et al*. 1999) or conversely how population level demographic processes influence selection on host resistance and pathogen infectivity loci.

Theoretical considerations of antagonistic interactions have produced two major competing genetic hypotheses describing coevolutionary processes: (1) arms-race dynamics occurring via selective sweeps of novel resistance and infectivity alleles and (2) fluctuating selection with oscillating allele frequencies maintained by negative frequency dependent selection (nFDS) (Leonard & Czochor 1970; Gandon *et al.* 2008; Brown & Tellier 2011). Evidence for an evolutionary arms-race favouring the emergence of new resistance and infectivity phenotypes comes mainly from molecular analyses of individual genes (Barrett *et al*. 2009; Paterson *et al*. 2010; Stukenbrock & McDonald 2009; Obbard *et al*. 2011; but see Stahl *et al.* 1999). Empirical studies focused on single populations and experimental microcosms have verified the potential for both nFDS and arms-race dynamics to drive coevolutionary change (Brockhurst *et al*. 2003; Decaestecker *et al*. 2007; Paterson *et al*. 2010; Gomez & Buckling 2011; Hall *et al*. 2011).

Direct evaluation of the relative impact of selective sweeps vs. fluctuating selection in naturally occurring host-pathogen metapopulations is largely still lacking (Dybdahl & Lively 1998; Paterson *et al*. 1998; Decaestecker *et al*. 2007). This is important because wild host-pathogen associations demonstrate considerable among-population demographic and genetic stochasticity [e.g. as a result of variable gene flow or the influence of environmental heterogeneity on disease epidemics (Smith *et al*. 2003; Soubeyrand *et al*. 2009)]. Verification of basic theory (van Valen 1973; Ebert 2008; Gandon *et al*. 2008) requires quantification of modes of coevolution in natural host-pathogen associations, the relative importance of different processes in the generation and maintenance of variation and the extent to which these might vary across years and populations.

Plant-pathogen interactions offer great potential for studying coevolutionary processes in natural systems. Plant immunity is mediated by germ-line encoded receptors that recognise specific pathogen components (Dodds & Rathjen 2010; Jones & Dangl 2006). Accordingly, many plant-pathogen interactions conform to the gene-for-gene model in which plant resistance (R) gene products (immune receptors) recognize pathogen effector proteins, classically known as avirulence (Avr) gene products, and trigger defence. The gene-for-gene paradigm implies the possibility of coevolution driven by selection pressure on pathogens to escape recognition by host R genes, and reciprocal pressure on hosts to respond to novel pathogen strains. The flax-flax rust interaction is a long-standing model for host-pathogen interactions spanning molecular, genetic, and population level studies (Dodds & Thrall

2009). The interaction between cultivated flax (*Linum usitatissimum*) and its fungal rust pathogen (*Melampsora lini*) was the basis of the gene-for-gene hypothesis and numerous corresponding R and Avr genes have been isolated in this pathosystem.

Melampsora lini also infects the Australian native flax (*L. marginale*), a naturally occurring association related to, but evolutionarily distinct from that between the pathogen and its agricultural host (Lawrence & Burdon 1989). Work on this system has centred on a set of local host and pathogen populations that interact within a larger metapopulation structure (Thrall *et al*. 2001, 2002). Previous studies demonstrate the basic ingredients of coevolution in that: i) *M. lini* has significant selective impacts on wild hosts, causing 60–80% reductions in population size during severe epidemics (Burdon & Thompson 1995); ii) host and pathogen populations exhibit considerable phenotypic diversity in patterns of resistance and infectivity (Thrall *et al*. 2001); iii) host resistance diversity can impact on the evolution of pathogen virulence, and disease epidemiology (Thrall & Burdon 2003); and iv) there is strong local adaptation by *M. lini* to its host (Thrall *et al*. 2002).

To investigate the potential for antagonistic coevolution to drive changes in patterns of resistance and infectivity in this natural host-pathogen metapopulation, we surveyed epidemiological dynamics of the pathogen between 2002 and 2008 across six local populations of *L. marginale* and *M. lini* in southeastern Australia. A comprehensive time-shift experiment (Gandon *et al*. 2008; Gaba & Ebert 2009) in which hosts and pathogens were challenged with sympatric past, contemporary and future counterparts, revealed temporal changes in resistance and infectivity. We directly investigated genetic change at interaction loci by genotyping pathogen populations for variation at two Avr genes (*AvrP123*, *AvrP4*) shown to elicit resistance reactions in *L. marginale* host lines from natural populations within the study area (Barrett *et al*. 2009). Finally, using an *Agrobacterium* transformation system to infiltrate plant leaves, we challenged host plants to determine their ability to recognize (i.e. resist) pathogen Avr gene variants that naturally occur in our study sites. Overall, our results reveal a complex mosaic of interactions that support the maintenance of population-level polymorphism by fluctuating selection.

EXPERIMENTAL METHODS

The pathosystem

Linum marginale (native flax) is a perennial herb endemic to southern Australia. In the subalpine Kiandra plain (Figure S1), plants overwinter as underground rootstocks with infrequent short shoots. Fresh shoots develop in spring and plants flower in mid- to late-summer before dying back in autumn. Within the Kiandra metapopulation, *L. marginale* plants are strongly inbreeding (Burdon *et al*. 1999).

Melampsora lini is a wind-dispersed rust fungus which undergoes 6–8 cycles of asexual spore production during the host growing season, leading to local epidemics. During the growing period, the pathogen is visible on living host tissue as localized (non-systemic) pustules. Following host die-back in the autumn, abrupt crashes in pathogen numbers occur. Although the rust is capable of initiating a sexual cycle, no evidence for sexual recombination has been detected in the Kiandra region (Barrett *et al*. 2008). The pathogen overwinters as limited numbers of dormant infections on occasional small green shoots, thus local extinctions are not uncommon.

In interactions with *L. usitatissimum*, *M. lini* exhibits complex infectivity polymorphisms controlled by up to 20 segregating loci, four of which have been cloned (Catanzariti *et al*. 2006). Alleles at two loci (*AvrP4*, *AvrP123*) have been shown to be differentially recognized by resistance genes in *L. marginale* (Barrett *et al*. 2009). Both loci exhibit strong adaptive

signatures (demonstrated by significant excesses of non-synonymous *vs*. synonymous nucleotide variation) and contain only single genes, facilitating population genetic analyses (Barrett *et al*. 2009).

Epidemiological surveys of populations and collections

At the beginning of the 2001–02 growing season, 6 *L. marginale* populations were selected within the Kiandra Plain metapopulation (Figure S1). During that season, and for each subsequent growing season up to and including 2007–08, each population was censused at 4–5 timepoints from the start of the season through to peak epidemic. The exception was 2006–07, when extensive bushfires made site access problematic and surveys were not conducted. In other years, at each timepoint, 100 plants haphazardly chosen from across the entire spatial area of each population were scored for disease status and infection severity (% tissue infected). At peak epidemic, 30–50 pathogen isolates (one per plant) were sampled per population. These were then inoculated onto seedlings of a universally susceptible cultivar (*L. usitatissimum* cv Hoshangabad). Approximately 5–7 days later, single pustules were isolated and put through 3 cycles of increase via sequential re-inoculation onto uninfected Hoshangabad seedlings to produce sufficient spores for inoculation studies. This procedure ensured that each isolate consisted of a single genotype. At the time of pathogen sampling, seed was collected separately from 30–50 plants haphazardly across each population irrespective of disease status.

Inoculation experiments

To characterize patterns of phenotypic change across years and within populations, we conducted a series of experiments using isolates collected during epidemic peaks. Following the 2007–08 growing season, individuals sampled from host and pathogen populations across the entire study period were evaluated using cross inoculation assays. Because of low disease prevalence and poor seed set in 2002–03 and bushfires in 2006–07, we performed this analysis using samples from every second season. Ten pathogen isolates were chosen from each population for 4 time periods (2001–02, 2003–04, 2005–06 and 2007–08) for a total of 40 isolates per pathogen population. Twenty host seedlings were chosen from each population x year combination (except for 2001–02 due to low seedset) for a total of 60 lines per host population. Within each population, pairwise inoculations were conducted across all pathogen x host x year combinations (2400 fully reciprocal pairwise inoculations per population, and 14,400 overall).

Shoots were cut from host plants and placed in water-filled tubs. Each tub included a cutting of the fully susceptible *L. usitatissimum* cv Hoshangabad to confirm pathogen viability. Tubs containing shoots of 14 host lines plus the control were inoculated with 10 mg of spores of a pure line of *M. lini*. The following day, tubs were transferred to a naturally lit greenhouse where infection was scored 12–14 days later. Infection types were: 1 = fully susceptible (large sporulating pustules (uredia) on all leaves); 2 = partial resistance (large sporulating pustules on younger leaves only, with no pustules on the oldest leaves); 3 = partial resistance (large pustules only on one or two of the youngest leaves); 4 = partial resistance (no sporulation, but with necrotic flecks on older leaves); 5 = full resistance (fully incompatible reaction with no macroscopic evidence of damage or sporulation). Infection type 1 plants were classified as susceptible; all other infection reactions were classified as resistant. Tests giving ambiguous results were repeated. These resistance phenotypes are all controlled by single dominant genes inherited in a simple Mendelian fashion (Burdon 1994).

To generate ‘multi-locus’ infectivity phenotypes, all pathogen isolates used in the glasshouse time-shift study were separately inoculated onto a ‘differential set’ of 11 standard *L. marginale* lines, each carrying different resistance genes or alleles. The resulting

pattern of resistant and susceptible responses across the host differential set provided a unique matrix that identified specific pathotypes (Figure S2).

Characterisation of AvrP4 and AvrP123 genotypes

Genotyping of *AvrP4* and *AvrP123* in isolates of *M. lini* was performed as per Barrett *et al*. (2009). Total genomic DNA was extracted from 100 mg of spores per isolate using a DNeasy 96 plant kit (QIAGEN). For *AvrP4*, a 568bp PCR product was amplified using 5′-CATCAAAATCTAACCCGTAC (forward) and 5′-GCACTATCTATCCTGAACAA (reverse) primer pair. PCR amplification consisted of an initial 3 minutes at 95°C followed by 34 cycles of 30s at 94°C, 30s at 56°C and 90s at 72°C. For *AvrP123*, a 598bp PCR product was amplified using 5′-ATTGTGAACCTTTTGAAGGAC (forward) and 5′-GCCATGGTATTGTTCAGAC (reverse). PCR conditions for *AvrP123* were similar to those of *AvrP4* with exception of 58°C annealing temperature. After PCR cleaning and BigDye™ Terminator Cycle Sequencing Reaction (PE Applied Biosystems), sequences were resolved on an ABI 3730XL automated sequencer. Alignments were performed using predicted protein sequences.

Transient expression assays for host resistance

Common *AvrP123* and *AvrP4* infectivity variants identified from the study sites (plus an additional previously identified variant, *AvrP123* Lm-5) were used to assess host resistance responses. Host populations K, N1, N2 and B3 were assayed for all years (2004, 2006, 2008). Populations B1 and B2 could not be evaluated across all years (2004 missing for B1, 2004 and 2006 missing for B2) due to poor survival subsequent to the glasshouse inoculation studies. In total 260 individual plants were assayed using DNA constructs encoding AvrP123 (lacking a signal peptide) and AvrP4 (including a signal peptide) protein variants controlled by the cauliflower mosaic virus 35S promoter (described in Barrett *et al* 2009). *Agrobacterium tumefaciens* (GV3101-pMP90) strains containing these constructs were prepared at an OD600 of 1.0 in 10mM MES pH 7.0, 10mM $MgCl_2$ buffer containing 200 μM acetosyringone and infiltrated into flax leaves. *A. tumefaciens* cultures containing an empty vector were used on all plants as a negative control. Each treatment was infiltrated into 12 leaves per plant, and 15–20 individuals per population were assayed. Resistance phenotypes were scored after 19–21 days, with necrotic responses interpreted as indicative of specific recognition. Individuals were excluded from analysis if more than 5 leaves infiltrated with cultures containing an empty vector exhibited a necrotic response (49 plants excluded). Plants were scored as positive for *AvrP123* or *AvrP4* recognition if the number of necrotic leaves was at least 3 more than the number of leaves infiltrated with an empty vector.

Statistical analyses

Analysis of infection data from the glasshouse inoculations was performed with GLIMMIX macro in SAS version 9.1 (SAS Institute 1999) using a GLMM with a nested design. Host response was a binary variable – resistant or susceptible – and hence, we used a logistic model with a binomial distribution of errors and a logit link function. Fixed categorical explanatory variables included population, host year (nested under population), pathogen year (nested under population), and their interaction (nested under population). Host lines and pathogen isolates were defined as random effects. To identify significant changes in pathogen infectivity and host resistance through time, contrast statements were used to compute all possible combinations of fixed model parameter means. To examine how pathogen populations vary in space vs. time with respect to the frequencies of infectivity phenotypes and Avr genotypes, we partitioned variance among populations and years using a permutation based multivariate analysis of variance (*adonis*) (Johnson 2001), using the package Vegan in R.

To determine links between pathogen population genetic structure and infectivity, frequencies of the four most common *Avr* variants (*Avr P123* Lm-1, *Avr P123* Lm-2, *Avr P4* Lm-1, *Avr P4* Lm-8) in the six pathogen populations in years 2002, 2004, 2006 and 2008 were regressed against infectivity of the corresponding pathogen population. Correlations between pairs of variants across loci suggest these may partly represent different clonal haplotypes (Figure S3), consistent with the largely asexual nature of these pathogen populations (Barrett *et al*. 2008). However, given differences in variant frequencies within years (Fig 1) and the potential for multiple haplotype combinations, we present analyses for individual variants, noting that results for combined haplotypes were essentially the same (Figure S4).

Infectivity estimates were obtained from the glasshouse inoculation experiment where infectivity of the pathogen population (ability to cause infection) was measured on host individuals from 2004, 2006 and 2008. We investigated the relationship between pathogen population genetic structure and disease epidemiology by regressing the frequency of these *Avr* variants in the six pathogen populations (years 2002, 2004, 2006, 2008) against data collected during the epidemiological surveys of infection prevalence. Regression analyses to compare Avr gene allele frequencies to infectivity and disease prevalence data were performed using proc reg in SAS Version 9.1 (SAS Institute 1999). Data on pathogen infectivity were normally distributed. Data on infection prevalence was square root transformed to meet assumptions of normality.

For the transient expression assays for host resistance, we first analysed separately host recognition of *AvrP123* and *AvrP4* variants, classifying individuals as recognising neither, one or both variants. We then analysed recognition of *AvrP123* and *AvrP4* together (i.e. how many of the 4 tested variants could be recognized by individual plants). We used host recognition of the variants as the explanatory variable and host resistance, as measured in the glasshouse inoculation trials, as the dependent variable. Host resistance was normally distributed. We therefore used ANOVAs to analyse the data as implemented by proc glm in SAS 9.1 (SAS Institute 1999).

RESULTS & DISCUSSION

Recurring disease epidemics in the *Linum-Melampsora* system

We first characterized temporal and spatial variability in the frequency and incidence of disease in wild populations of *L. marginale*. Field surveys conducted between 2002 and 2008 showed that, over the study period, disease prevalence at peak epidemic was high in most populations, although there was variation among host populations within single growing seasons and across years (Table S1). For example, more than 80% of plants in populations B1, B2 and B3 were infected in 2004, 2006 and 2008, while infection levels generally declined across the period in population K, and all populations showed low levels of infection in 2002–03 (Table S1), likely due to drought. Together with previous studies (Burdon & Thompson 1995; Thrall *et al*. 2001, 2002; Barrett *et al*. 2008, 2009), this demonstrates that our system meets basic ecological and genetic requirements for coevolutionary change, in that diverse *L. marginale* hosts are regularly parasitized in a genotype-specific manner by diverse, debilitating pathogens.

Populations undergo extensive and rapid changes in resistance and infectivity

The two mechanisms most often proposed to underlie host-parasite coevolution are fluctuating selection (via nFDS) and arms-race dynamics (via selective sweeps). Predictions for patterns of genetic change within populations differ depending on which mechanism is predominant (Gandon *et al*. 2008). If coevolutionary change were driven by selective

sweeps, pathogens from the past should be less infective on contemporary hosts than contemporary pathogens, while pathogens from the future should be more infective on hosts from the past (Gandon *et al.* 2008; Gaba & Ebert 2009). Alternatively, if coevolution were dependent on nFDS, then predictions regarding the direction of genetic change depend on when samples are taken in the coevolutionary cycle. Thus, in a metapopulation context where local populations are likely cycling out of phase (Thrall *et al*. 2002), past pathogens may be predicted to have higher infectivity in some subpopulations but lower infectivity in others.

To investigate whether genetic change in the *Linum-Melampsora* system is consistent with expectations arising from either model, we performed a time-shift inoculation experiment, where host plants from each population were exposed to their contemporary, past and future sympatric pathogen isolates (Gandon *et al*. 2008; Gaba & Ebert 2009). The results demonstrated significant differences in host resistance and pathogen infectivity across years within individual populations, as well as overall differences among populations (Table S2). To identify potentially adaptive changes, we examined shifts in host resistance using the corresponding past pathogen population and shifts in pathogen infectivity using the corresponding contemporary host population (Fig 2). For example, a change in pathogen infectivity from 2004–06 was measured by comparing pathogens from 2004 and 2006 on hosts from 2006. An analogous change in resistance was measured as the difference in infectivity of pathogens from 2004 on hosts from 2004 and 2006. Our data reveal complex and multi-directional changes in resistance and infectivity over time (Fig 2). Of the 30 temporal comparisons, nearly half (13) showed statistically significant changes in host resistance or pathogen infectivity, with significant changes recorded in every population (Fig 2b, Table S2).

We found two types of temporal change: those of likely adaptive value (increases in infectivity caused by changes in pathogen populations and decreases in infectivity caused by changes in host populations; 8 in total) and those of seemingly counter-adaptive value (decreases in resistance and infectivity; 5 in total). However, the observed changes did not follow clearly predictable trajectories and coevolutionary processes were often asynchronous among populations. In particular, results from these experiments did not reveal net increases in pathogen infectivity, or decreases in host resistance, over time (Fig 3). For example, in 2004, hosts from populations B2 and B3 were less resistant to contemporary pathogens than to either past or future pathogens, while in population N1 essentially the reverse was observed; N1 hosts in 2006 were most resistant to their contemporary pathogens (Fig 3). We conclude that the constant escalations in resistance or infectivity predicted by a coevolutionary arms-race model are not occurring over the ecological timescales represented by this study. This is consistent with recent results from experimental studies of coevolution between *Pseudomonas fluorescens* and phage which suggest that, in antagonistic interactions, fluctuating selection is more likely to be maintained over time than arms-race dynamics (Gomez & Buckling 2011; Hall *et al*. 2011).

Instead we argue that the complex shifts in patterns of infectivity and resistance we observed better reflect populations cycling through different phases of nFDS (Gandon et al. 2008). In three of the six host-pathogen populations (N1, B1, B2), there was significant evidence of adaptive change in infectivity and resistance (Fig 2, Table S2). In these populations an increase in pathogen infectivity is followed by an increase in host resistance (e.g. in population B2 pathogen change from 2002 to 2004 is followed by a change in the host from 2004 to 2006), and an increase in host resistance drives an increase in infectivity of the respective pathogen population (e.g. pathogen population N1 from 2006 to 2008). We also observed significant decreases in infectivity (pathogen population B3 from 2002 to 2004, population K from 2004 to 2006; Fig 2) which would not be predicted under an arms-race

scenario. Moreover, population level surveys indicate persistent fluctuations in frequencies of common pathogen infectivity phenotypes (Figure S2) and genotypes (Fig 1) rather than emergence and spread of new variants. These data are thus generally consistent with expectations for genetic change via reciprocal cycling in host resistance and pathogen infectivity (Sasaki 2000), with high levels of host resistance in a previous year selecting for higher pathogen infectivity and vice-versa.

However, as might be expected in spatially structured natural systems (Gomulkiewicz *et al*. 2000), other populations exhibited patterns difficult to reconcile with either the arms-race or fluctuating selection models. In populations B3 and N2, large shifts in infectivity and resistance of contemporary host pathogen combinations were driven by changes in only one partner (Fig 2b, Table S2). Importantly, these changes were not unidirectional. In B3 for instance, pathogen infectivity decreased from 2002 to 2004, and then increased in 2006. We also found evidence that resistance may decrease in host populations following lower pathogen infectivity (e.g. change in host population N1 from 2006 to 2008; Fig 2b), raising the possibility of a cost of resistance (Tian *et al*. 2003). Finally site K showed little change in infectivity or resistance across the time-shift experiment suggesting this population might represent a coevolutionary 'coldspot'. Consistent with this lack of change, survey data indicated that host numbers and disease prevalence in this population generally declined over the 2002–08 study period and in 2008, there was a major epidemic that occurred in all populations except K (Table S1).

The importance of including multiple populations in coevolutionary studies is further evidenced by results from a permutation-based multivariate analysis of variance which partitioned variation in the frequencies of pathogen infectivity phenotypes and Avr genotypes among populations and years. These analyses showed that while there was a significant main effect of year, a much greater proportion of the observed variance in infectivity could be explained by the main effect of population and the population by year interaction (Table S3, Figure S2). Together, these data emphasize that simple theoretical models of coevolution are likely insufficient to predict phenotypic changes in resistance or infectivity in wild host-pathogen associations involving multiple loci, and highlight the need for multiple spatial and temporal samples in analyses of such systems (Gandon *et al*. 2008). While a significant proportion of observed changes in resistance and infectivity are consistent with evolution via fluctuating selection, other results suggest that the influence of selection on patterns of resistance and infectivity may be strongly mediated by spatial and temporal heterogeneity in patterns of disease incidence, gene flow and genetic drift (Brockhurst *et al*. 2003; Thompson 2005).

Changes in infectivity and resistance correspond to shifts in genotype frequencies in pathogen and host populations

Gene-for-gene systems are characterized by interactions between resistance and infectivity genes across multiple loci (Dodds & Thrall 2009). If these loci are not cycling in phase, then temporal stability in average resistance and infectivity may mask underlying changes in the frequency and identity of individual resistance and infectivity alleles. To directly investigate temporal changes at loci underlying phenotypic variation in infectivity, we surveyed population genetic variation at two effector loci (*AvrP123*, *AvrP4*), alleles of which differentially trigger resistance responses in *L. marginale* hosts. Previous population analysis of patterns of nucleotide variation at AvrP123 and AvrP4 showed high polymorphism and a strong signature of diversifying selection (Barrett *et al*. 2009), implying adaptive change at the population level. Sequencing identified twenty-five alleles of *AvrP4* across the 240 pathogen isolates sampled from the 6 host populations. Of these, 23 represented rare genotypes that were only found once. Two previously described alleles *of AvrP4*, Lm-1 and Lm-8 (Barrett *et al*. 2009), were found in 90% of the isolates. For

AvrP123, 85% of the sequences could be readily resolved – the remainder of the isolates appeared to carry multiple copies at this locus. Again, two variants, *AvrP123* Lm-1 and *AvrP123* Lm-2 accounted for 99% of the isolates in the resolved sample. Thus, pathogen populations were dominated by two common genetic variants at each locus. Consistent with our phenotypic data, their frequencies fluctuated substantially across populations and years (Fig 1, Table S3).

Importantly, our data demonstrate that the frequencies of major Avr variants correlate strongly with overall infectivity observed in the time-shift experiments. The alleles *AvrP4* Lm-1 and *AvrP123* Lm-1 showed a strong negative correlation with infectivity (Fig 4a,c), suggesting that pathogen isolates containing this combination of allelic variants were likely to be less infective overall. The opposite correlation with infectivity was found for the other pair (*AvrP4* Lm-8 and *AvrP123* Lm-2; Fig 4b,d). Consistent with this, the average infectivity of pathogen isolates carrying *AvrP123* Lm-1 and *AvrP4* Lm-1 differed significantly from those containing *AvrP123* Lm-2 and *AvrP4* Lm-8 (46.9% and 59.0% respectively; $F_{1,172} = 17.256$, $P<0.0001$).

Although the R genes underlying resistance specificities in *L. marginale* have not been identified, host plants can differentially recognise alleles of the pathogen effector molecules *AvrP123* and *AvrP4* (Barrett *et al*. 2009). Thus, the infiltration studies we conducted using *Agrobacterium*-mediated transient expression allowed us to pinpoint changes in the frequency of resistance specificities in *L. marginale* populations. Host lines from each population and year evaluated in the inoculation study were challenged with Avr gene variants identified as common from earlier surveys of wild *M. lini* populations within our study area (Barrett *et al*. 2009). Three variants (*AvrP123* Lm-1, *AvrP4* Lm-1, *AvrP4* Lm-8) generally dominated the pathogen populations evaluated in the current study. The fourth (*AvrP123* Lm-5) was chosen because it represented a distinctly different recognition pattern on *L. marginale*.

Recognition of these variants, as indicated by cell death induction (Fig 5a) occurred in all host populations, but frequencies varied considerably among populations and years (Fig 5b–e). Hosts exhibiting necrotic reactions to one or both *AvrP123* variants in the transient expression assay also showed significantly higher levels of phenotypic resistance in the glasshouse inoculation trial than individuals showing no reaction ($F_{2, 137} = 5.33$, $P = 0.0059$; Fig 5f) and there was a marginally significant negative relationship between the frequency of *AvrP123* recognition and disease prevalence during naturally occurring epidemics at our study sites ($R^2 = 0.279$, $P = 0.077$). Overall, this argues that recognition of *AvrP123* contributes to host resistance within the Kiandra region. In contrast, no relationship was observed between *AvrP4* recognition and host resistance ($F_{2, 137} = 2.82$, $P = 0.7547$), implying that recognition of this locus in the host may not strongly influence disease outcomes in these populations. This is not unexpected – it is likely that interactions between multiple corresponding R-Avr loci contribute to variation in patterns of resistance and infectivity in nature (Dodds & Thrall 2009). Many potential effectors have been identified in *M. lini* (Catanzariti *et al*. 2006) and there is no *a priori* reason to expect that all such loci will affect the interaction in a given subset of host populations during a particular time period.

Infectivity costs influence epidemiological outcomes in nature

We observed that the frequency of specific Avr alleles correlates with the average infectivity of pathogen populations (Fig 4a–d) and that host recognition of these variants correlates with overall resistance (Fig 5f). This indicates that temporal shifts in resistance in some populations (Figs 2, 3) can be explained by changes in the frequency of recognition of infectivity variants in the corresponding pathogen population. Such changes in host

resistance or pathogen infectivity could in turn drive variation in disease levels during natural epidemics (Table S1; Thrall & Burdon 2000). Thus, during naturally occurring epidemics in the wild host populations, we found highly significant relationships between the frequency of specific pathogen variants and disease prevalence (Fig 4e–h). Interestingly, these relationships were in the opposite direction to the correlations with infectivity (Fig 4a–d), meaning that when highly infective genotypes dominated pathogen populations, significant reductions in disease were observed (Fig 4f,h).

These findings are consistent with a previously demonstrated trade-off between aggressiveness (spore production) and infectivity, and observations that highly infective pathotypes do not dominate susceptible host populations in the *Linum-Melampsora* system (Thrall & Burdon 2003). Together, these results imply that shifts in pathogen infectivity mediated by the observed trade-off can influence levels of disease in nature. This hypothesis is further supported by the fact that we find only marginal correlations between disease prevalence and the population frequencies of hosts able to recognise these variants. This is despite the fact that host recognition (at least for *AvrP123*) is significantly correlated with overall resistance as measured in the inoculation studies. While reductions in disease prevalence may also be driven by increased resistance in host populations, even in highly resistant populations there are always pathotypes present that can cause disease (Thrall & Burdon 2003). Moreover, as our data show, less infective isolates carrying *AvrP123* Lm-1 or *AvrP4* Lm-1 do not go extinct (Fig 1). Theoretical studies also show that maintenance of pathogen variants with different infectivity levels is likely to partly depend on costs associated with antagonistic pleiotropy (Leonard & Czochor 1970; Salathé *et al*. 2005). Importantly, variation in the strength of such trade-offs can strongly influence expectations for balancing selection in multi-locus gene-for-gene interactions, with complex patterns of cycling predicted to occur under some conditions (Sasaki 2000).

Conclusions

Comprehensive glasshouse inoculations across multiple populations and years demonstrate significant temporal shifts in pathogen infectivity and host resistance. The inference that antagonistic interactions drive temporal variation in infectivity and resistance via fluctuating selection is strongly supported by corresponding changes in the frequencies of common allelic variants of infectivity genes, and host recognition of these variants. Critically, we demonstrate direct links between these genetic changes and disease prevalence in nature. We observed several examples of antagonistic cycling between pathogen infectivity and host resistance, consistent with nFDS, but did not detect evidence for selective sweeps consistent with an arms-race scenario. Many of these patterns have been documented experimentally under controlled conditions for other antagonistic associations (Brockhurst *et al*. 2007; Poullain *et al*. 2008; Koskella & Lively 2009; Paterson *et al*. 2010). Although clearly more complex, analysis of wild host-pathogen interactions is essential to link results from experimental systems to natural situations. As encapsulated by the Geographic Mosaic Theory of Coevolution (Gomulkiewicz *et al*. 2000; Thompson 2005), the considerable spatial variation in these interactions, as well as in the strength of local coevolutionary dynamics, should perhaps be expected. Such findings accord with models which demonstrate that, despite the potential for epidemiological and evolutionary complexity, coevolution in spatially structured host-pathogen associations can maintain significant levels of polymorphism in resistance and infectivity (Thrall & Burdon 2002; Brockhurst *et al*. 2003; Laine & Tellier 2008).

In sum, this study represents the most comprehensive empirical evidence to date of the importance of coevolutionary processes in maintaining variation in a wild host-pathogen association. Theory on antagonistic interactions has emphasised the critical role that spatial structure, frequency dependent change and trade-offs play in driving coevolutionary

outcomes. However, the restriction of previous empirical studies to in vitro experiments, single populations or single timepoints has limited the extent to which inferences regarding coevolution in natural situations can be drawn. By revealing temporal patterns of genetic change in a wild metapopulation, our work provides support for much of the theory underlying our understanding of coevolutionary change, and further confirms the role of infectivity costs in the population dynamics of antagonistic associations. Perhaps more importantly, this study also demonstrates that, despite the complexities inherent in interpreting data from spatially variable natural host-pathogen associations, it is indeed possible to identify the signature of coevolution in such systems, and to directly link epidemiological patterns to both phenotypic and genetic change.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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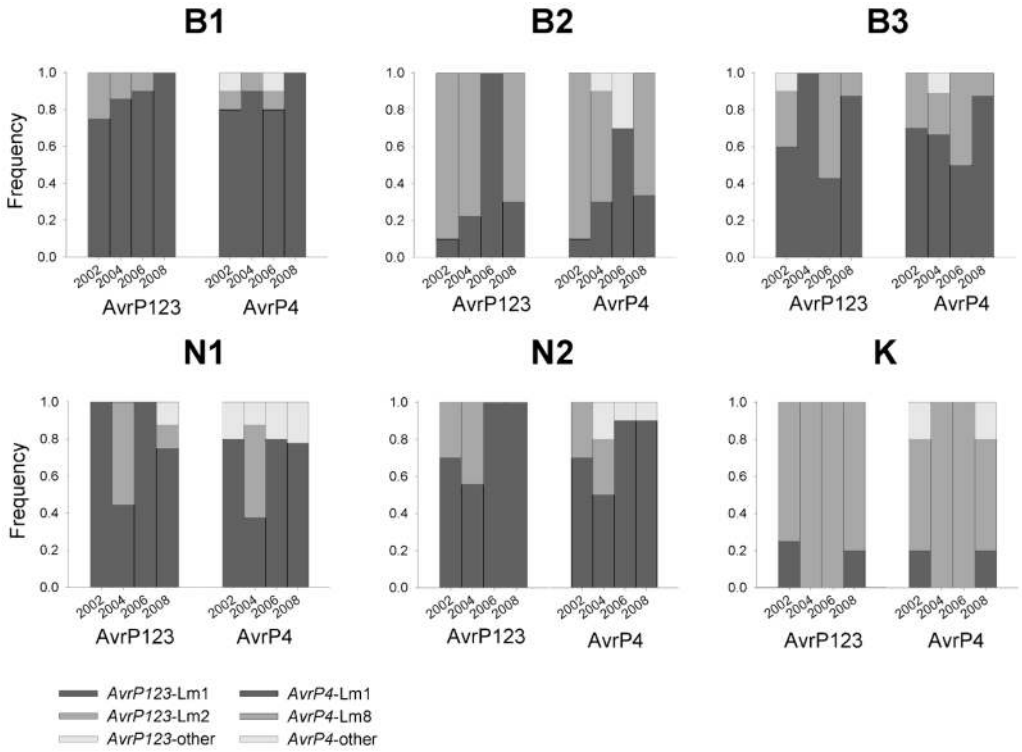

Figure 1.

Variation in infectivity gene frequencies across years and populations. Sequence analysis of variation at two infectivity loci (*AvrP123*, *AvrP4*) was carried out on pathogen isolates used in the glasshouse inoculation study. Two common variants at each *Avr* locus were identified (*AvrP123*: Lm-1, Lm-2; *AvrP4*: Lm-1, Lm-8), although a number of other variants were detected at low frequencies. Frequencies of these variants are shown for each population across years, from 2002 through to 2008.

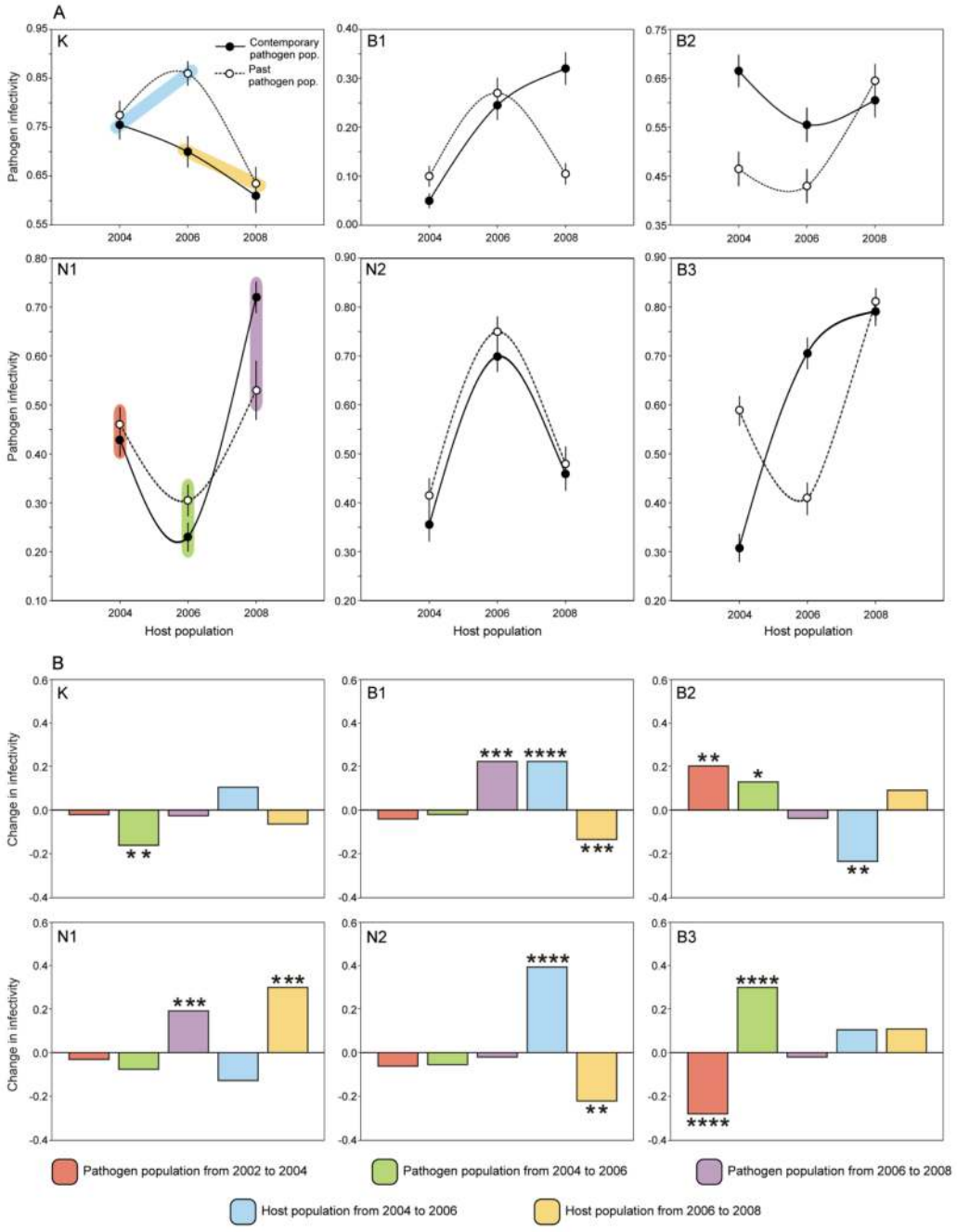

Figure 2.

Variation in pathogen infectivity and host resistance as assessed by glasshouse inoculations. (A) Hosts from 2004, 2006 and 2008 were evaluated against sympatric pathogens from 2002, 2004, 2006 and 2008. Infectivity/resistance (lower infectivity = increased resistance) is shown for contemporary host and pathogen combinations (filled circles) and for hosts infected with pathogens from two years previously (open circles). Color shading in the K and N1 graphs illustrates points used to evaluate cross-year changes in resistance (◆=2004–2006; ◆=2006–2008) and infectivity (◆=2002–2004; ◆=2004–2006; ◆=2006–2008). (B) Changes in infectivity and resistance between consecutive time periods (see text for details).

Significant changes are highlighted (*<0.05, **<0.01, ***<0.001, ****<0.0001; see Table S1).

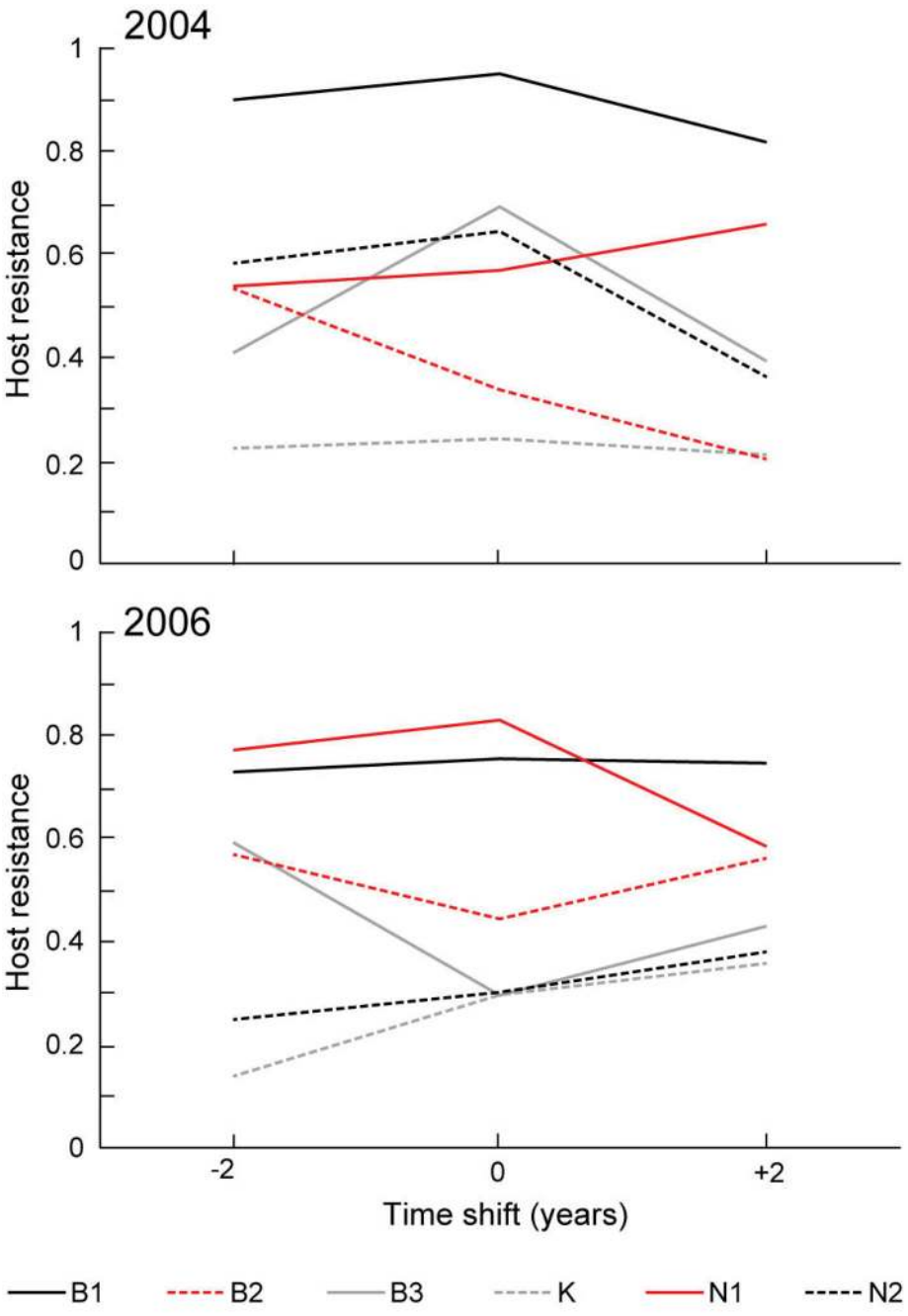

Figure 3.
Changes in pathogen infectivity over time. The infectivity of past, contemporary and future pathogen populations on their sympatric host populations from 2004 (upper panel) and 2006 (lower panel) is plotted.

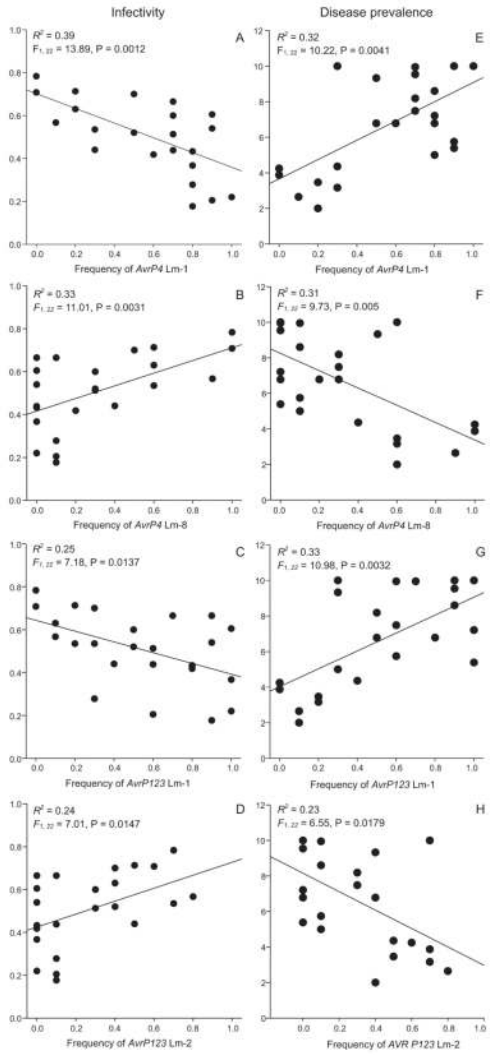

Figure 4.

Frequencies of common pathogen variants for two infectivity loci, *AvrP123* and *AvrP4*, regressed against: a–d) their average infectivity as calculated from the glasshouse inoculation data; and e–h) disease prevalence scored during natural epidemics in the wild host populations for the same years. Variant frequencies were based on molecular analysis of the pathogen isolates collected from hosts infected in the wild (the same isolates used in the glasshouse inoculation trials). Average infectivity was determined using the scores across all years for a given population. Disease prevalence was square-root transformed prior to regression analyses to improve normality (see Methods).

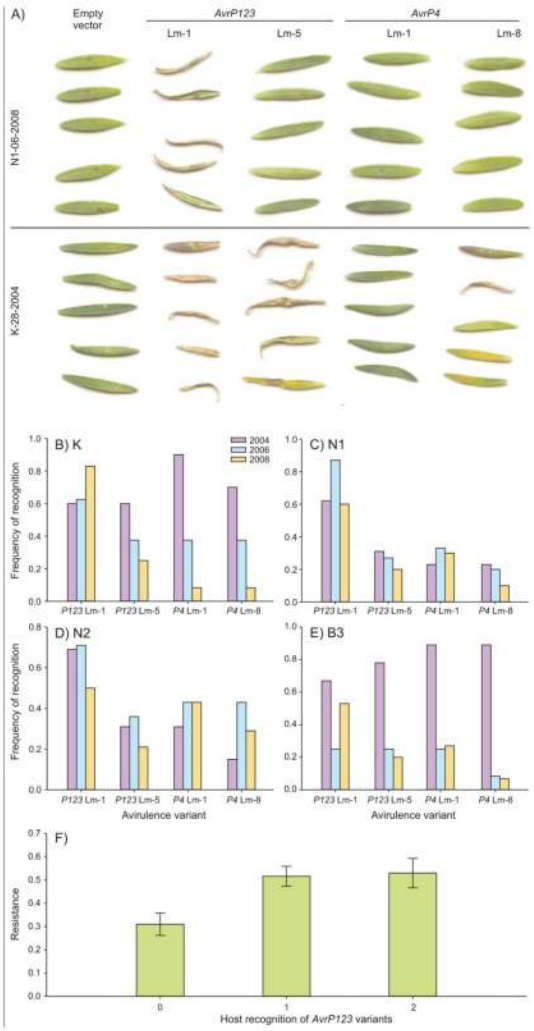

Figure 5.

Host lines used in glasshouse inoculations were evaluated for their ability to mount necrotic responses to infiltration with variants of two *Avr* genes (*AvrP123*, *AvrP4*). (A) *L. marginale* lines N1-06-2008 and K-28-2004 display differential necrotic responses following infiltration with *Agrobacterium* cultures containing T-DNA plasmids encoding *AvrP123* alleles Lm-1 and Lm-5, and *AvrP4* alleles Lm-1 and Lm-8. (B–E) Variation in the frequency of resistance reactions to these variants for a subset of host populations for which full data were available. (F) Analysis of the relationship between recognition of *AvrP123* alleles and resistance (based on glasshouse inoculations). Hosts showing necrotic reactions to one or both *AvrP123* variants were significantly more resistant than hosts that didn't react ($F_{2,\ 137}$=5.33, P=0.0059).