

## ORIGINAL ARTICLE

## Rapid evolution in a plant-pathogen interaction and the consequences for introduced host species

Gregory S. Gilbert<sup>1</sup> and Ingrid M. Parker<sup>2</sup><sup>1</sup> Environmental Studies Department, University of California, Santa Cruz, CA, USA<sup>2</sup> Ecology and Evolutionary Biology, University of California, Santa Cruz, CA, USA**Keywords**

biological invasions, biotic resistance, clovers, escape from natural enemies, host range of plant pathogenic fungi, *Medicago*, rapid evolution, *Trifolium*.

**Correspondence**

Gregory S. Gilbert, Environmental Studies Department, 1156 High St., University of California, Santa Cruz, CA 95064, USA.  
Tel.: (831) 459-5002;  
fax: (831) 459-4015;  
e-mail: ggilbert@ucsc.edu

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**Abstract**

Plant species introduced into new regions can both leave behind co-evolved pathogens and acquire new ones. Traits important to infection and virulence are subject to rapid evolutionary change in both plant and pathogen. Using *Stemphylium solani*, a native foliar necrotroph on clovers (*Trifolium* and *Medicago*) in California, USA, we explore how plant-fungal interactions may change in an invasion context. After four generations of experimental serial passage through multiple hosts, *Stemphylium* consistently showed increased infection rates but no consistent change in damage to the host. In a historical opportunity study, we compared infection and virulence across four groups of clover hosts: California natives, European clovers not found in California, and both California and European genotypes of species naturalized in California. There was significant variation among hosts, but no pattern across the four groups. However, in direct comparisons of familiar California genotypes to unfamiliar European genotypes of the same naturalized species, *Stemphylium* consistently infected familiar hosts more frequently, while causing less damage on them. This pattern is consistent with the hypothesis of adaptive evolution in both the pathogen (ability to infect) and the host (tolerance of infection). Together these results suggest the potential for rapid evolution to alter interactions between plant invaders and their natural enemies.

**Introduction**

*Getting to know you, getting to know all about you.*

Oscar Hammerstein

A central hypothesis of invasion biology is the idea that introduced species leave their natural enemies behind in their home range and are released from pest pressure relative to the native species with which they compete (Elton 1958; Gillett 1962; Maron and Vilá 2001; Keane and Crawley 2002; Colautti et al. 2004). For the case of plants and their pathogens, some studies have provided evidence for such 'Escape from Natural Enemies' (Wolfe 2002; Mitchell and Power 2003; Agrawal et al. 2005) while others have questioned its importance (Blaney and Kotanen 2001; Beckstead and Parker 2003; Parker and Gilbert 2007; van Kleunen and Fischer 2009).

Evolution has been suggested to play a role in the release from natural enemies. The EICA (Evolution of Increased Competitive Ability) Hypothesis suggests that evolution of plants away from investment in defense might allow populations of introduced species to allocate more resources toward competitive ability (Blossey and Notzold 1995). There is some empirical evidence that genotypes of introduced species from the new range can grow faster, are more fecund, or are more poorly defended than those from the native range (Siemann and Rogers 2001; Bossdorf et al. 2005), whereas other studies have failed to find such evidence (Willis et al. 2000; Maron et al. 2004). Interestingly, these discussions of evolution of introduced species assume that the populations of local natural enemies do not themselves evolve in response to the availability of new hosts. While we

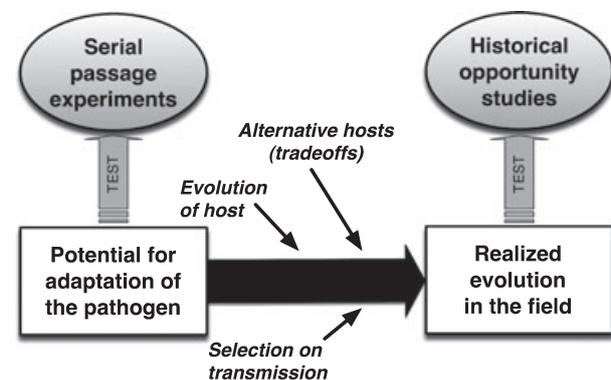
currently have no clear examples of herbivores or pathogens that have shifted onto introduced hosts through evolutionary processes (i.e., requiring genetic change), there are many cases of ecological host shifts, which then might be followed by adaptation of the herbivore or pathogen to utilize those hosts more effectively (reviewed in Parker and Gilbert 2004; e.g., Carroll et al. 2005). Pathogen infectivity may change over time through frequency-dependent selection (the Red Queen Hypothesis) (Clay and Kover 1996; Thrall et al. 2002; Dybdahl and Storer 2003). Even for polyphagous pathogens (those pathogenic on multiple local host species), the ability to infect a particular host species may change over time in response to variation in the relative abundance of different host species in the local plant community (Parker and Gilbert 2004). Therefore, as an invading host becomes more abundant in the community (and more frequently encountered by the pathogen), we expect an increase in the frequency of those pathogen genotypes most able to infect and reproduce on the dominant host species, leading to increased disease incidence or severity. Virulence (the degree of damage caused by a pathogen on a particular host) may decrease when it is negatively correlated with transmission (May and Anderson 1983; Herre 1993; Frank 1996; Lipsitch et al. 1996). In contrast, virulence of necrotrophic or polyphagous pathogens may instead increase when damage to the host increases pathogen fitness (Jarosz and Davelos 1995). Given the disproportionately rapid generation times of most microbes, we should expect that evolutionary change on the part of natural enemies could rather quickly modulate the benefit of evolutionary reallocation away from defense in the host.

We still know relatively little about the evolutionary dynamics of plant-pathogen interactions in natural systems (Jarosz and Davelos 1995; Roy and Kirchner 2000; Gilbert 2002; Burdon et al. 2006). However, the agricultural literature provides us with a rich source of information about both short-term and relatively long-term evolutionary change in response to changes in the distribution and availability of specific host species or genotypes. In serial passage experiments, a pathogen is passed for multiple generations through the same host species or genotype. Because the hosts are usually particular crop varieties kept constant throughout the experiment, these experiments test specifically whether and how rapidly a pathogen can adapt to a host that is kept genetically constant. Serial passage experiments have demonstrated that evolution occurs rapidly in some systems (e.g., Jinks and Grindle 1963; Harrower 1977; Ruffy et al. 1981; Groth and Ozmon 1994) but not others (e.g., Fitzgerald and Cooke 1982; Rozycki et al. 1990). Because most serial passage experiments utilize highly homozygous and uniform

domesticated varieties, it is not clear whether pathogens could evolve as rapidly when passed through genetically diverse populations of wild plants.

Serial passage experiments can only provide an indicator for a limited part of the scope of evolutionary dynamics of plant-pathogen interactions (Fig. 1). In serial passage experiments, transmission is ensured by the researcher, and therefore selection on the pathogen acts only on within-host fitness and not on between-host fitness. In addition, the host is not allowed to evolve in response to the pathogen. Incorporating these other factors could obviously greatly change the dynamics of the pathogen. A different approach is taken in 'historical passage' studies, which do not utilize experimental treatments but rather take advantage of historical opportunities such as the introduction of a particular crop variety to compare the genetic makeup or behavior of a pathogen from locations planted with that crop variety to the behavior of that same pathogen from other sites (Tjamos 1981; Lindemann et al. 1984; Kolmer 1991; Hovmoller et al. 1993). Sometimes these studies track temporal changes directly (Simons and McDaniel 1983; Kolmer 1989; Boshoff et al. 2002) and sometimes evolution is inferred from comparisons at the landscape scale (Lindemann et al. 1984; Hovmoller 2001).

The introduction of non-native plant species provides us with this type of 'historical opportunity' to study the



**Figure 1** The potential for a pathogen to adapt to a new host can be tested using serial passage experiments in the greenhouse. That potential may not, however, lead to adaptive evolution of the pathogen to that host in a field setting. Factors such as selection on between-host transmission, tradeoffs in the ability to infect different hosts, and the evolution of host resistance in response to the pathogen may all moderate evolutionary outcomes. One way to test for that realized evolution is through historical opportunity studies: e.g., comparing interactions between 'familiar' genotypes (e.g., a native pathogen with an introduced host with which it has coexisted for several decades) to interactions between 'unfamiliar' genotypes (e.g., the native pathogen with that same host species but on genotypes collected from the native range).

outcomes of evolution in host-pathogen interactions over intermediate time scales in natural systems. We can learn a lot about the potential for evolution to influence the interactions between invasive plants and their natural enemies by studying the behavior of pathogens on hosts with which they share different degrees of evolutionary history, or 'familiarity'. In this study we used a common, generalist pathogen and a suite of native and non-native hosts as a model for assessing the potential for rapid evolution on individual host species, and whether patterns of host use are consistent with the hypothesis of adaptive evolution in the interaction between host and pathogen since time of introduction.

The clover genus *Trifolium* has two centers of high diversity – Mediterranean Europe (~119 native species) and California in the United States (~56 California native species). In addition, the closely related burr-clover genus *Medicago* has its center of diversity in Mediterranean Europe and North Africa (~39 species) [species estimates from accepted and provisional species listed in the online databases of Jepson Online Interchange (<http://ucjeps.berkeley.edu/interchange.html>) and Flora Europaea (<http://rbg-web2.rbge.org.uk/FE/fe.html>) on 31 May 2009]. Numerous species of both *Trifolium* and *Medicago* from Europe have been introduced into North America (18 and 10, respectively, in California), and many have escaped cultivation and are widespread both as ruderals in anthropogenic habitats and as invaders into wild habitats. In the coastal prairie at one 1.5-km<sup>2</sup> site at the Bodega Marine Reserve (BMR) on the central California coast, 10 native species of *Trifolium* are joined by nine introduced species of *Trifolium* and *Medicago* that have been in California for eighty to hundreds of years (Parker and Gilbert 2007). One fungal genus, the Ascomycete *Stemphylium* (anamorph of *Pleospora*), is the most important cause of leaf necrosis and early senescence of clovers at BMR, and is by far the most dominant foliar fungus (Parker and Gilbert 2007).

Here we present the results of both a serial passage experiment and a historical opportunity study to examine how a pathogen may change in response to novel hosts. In the serial passage experiment, we evaluated the potential for rapid adaptation to novel hosts by comparing levels of infection and virulence (i.e., necrosis and leaf senescence) of two independent strains of the California native fungal pathogen *Stemphylium solani* to that observed after each fungal strain was passed through four generations on each of eleven clover hosts. We expect that with serial passage, infection ability should increase and virulence should also increase (because there is no inherent tradeoff with between-host fitness). In the historical study, we compare the infection ability and virulence of the pathogen strains on three species of

California native clovers, three species of European clovers never before introduced to California, and three species of clovers naturalized in California, including both genotypes collected in California and genotypes collected from Europe. We predict that infection ability of *Stemphylium* will be highest on the host species with which it is most 'familiar' and lowest on the completely novel European species. We predict that virulence will also be greater on familiar hosts, because the pathogen sporulates on dead plant tissue. When familiar genotypes are compared to unfamiliar genotypes of the same species, the pathogen should be most effective at exploiting the genotypes with which it shares an evolutionary history.

## Materials and methods

### Plants and fungi used in the experiments

We examined how infection and virulence of two California strains of the plant pathogenic Ascomycete fungus *S. solani* G.F. Weber (anamorphic form of *Pleospora*) varied across 12 clover lineages. Host species were chosen from a range of *Trifolium* and *Medicago* species (hereafter referred to as 'clovers') occurring in California, USA and in Mediterranean Europe. We used four sets of hosts: three species of clovers native to the coastal prairie at the University of California Bodega Marine Reserve in central California, USA (Native-CA: *T. gracilentum* Torr. & A. Gray, *T. fucatum* Lindl., *T. macrei* Hook. & Arn.); three species native to southern France that had never been introduced into California (Novel-EU: *T. cherleri* L., *T. striatum* L., *M. trunculata* Gaertn.); three species native to southern Europe but naturalized in California (Exotic-CA: California-collected seeds of *M. polymorpha* L., *T. campestre* Schreb., *T. glomeratum* L.); these same three exotic species, but with seeds collected from their native range (Exotic-EU). Seeds for Native-CA and Exotic-CA species were collected in bulk from dozens of maternal plants from the Bodega Marine Reserve in Bodega Bay, California, USA (BMR: 38.3363°N, 123.04635°W), and seeds for Exotic-CA and Exotic-EU lineages from Els Banys, Var, France (42.36849°N, 1.59479°E).

The pathogen we used was the North American native pathogen *S. solani*, the most common foliar endophytic fungus found in clovers at the BMR site (Parker and Gilbert 2007). We determined the identity of *S. solani*-based morphological characteristics and through comparison of rDNA ITS 1 and 2 sequences with a published systematic treatment of *Stemphylium* (Cámara et al. 2002; Parker and Gilbert 2007). Comparison of 51 ITS sequences of our own European collections and worldwide GenBank accessions of *Stemphylium* spp. plus 65 published records of *S. solani* in the USDA fungal databases (Farr et al.

2006) both suggest that *S. solani* is absent from Europe but is widespread in, and presumably native to North and South America (Parker and Gilbert 2007). It causes leaf necrosis and senescence, but can also be found in apparently healthy leaves. *Stemphylium* spp., primarily *S. solani*, comprised 64% of 1175 fungal isolates collected from clovers over 4 years at BML (Parker and Gilbert 2007). We used two single-mitospore strains of *S. solani* to evaluate infectivity and virulence on clover hosts: strain barb34L (GenBank accession EF104156) was isolated from *T. barbigerum*, and bifi4C (GenBank accession EF104157) from *T. bifidum*. Both strains were isolated from necrotic lesions on diseased leaves on clovers growing in the coastal prairie at the Bodega Marine Reserve. Both original host plants are California natives, but were not among the species included in the experiments.

### Inoculum preparation

Inoculum was produced by growing cultures on V8-juice agar (82 mL V8 juice, 0.9 g CaCO<sub>3</sub>, 7.5 g agar, 371 mL water) in 9-cm Petri dishes under ambient laboratory conditions. After 2–3 weeks, 30 mL sterile deionized water was poured onto the culture, and spores were dislodged by rubbing the colony surface with a sterile rubber policeman. The suspension was poured through a double-layer of cheesecloth into a sterile 50-mL Falcon centrifuge tube. Spore concentration was quantified for each suspension by counting with a hemacytometer (mean  $9300 \pm 3400$  spores mL<sup>-1</sup>).

### Serial passage procedure

To evaluate whether there was cross-generational adaptation to particular host lineages, we passed each of the two *S. solani* strains (barb34L and bifi4C) through four generations of transmission within each of the 12 clover lineages. We began with a single-spore isolate of each strain (hereafter, Generation 1). We grew three individuals of each of the clover lineages for 6–10 weeks, randomized in a growth chamber (14 h daylight, 20°C in the day, 15°C at night, 60% humidity, and two banks of lights). If necessary, we trimmed the plants back to 8–20 leaves before inoculating. Three individuals of each of clover lineage were inoculated with each fungal strain, by dipping all leaves on a plant into a spore suspension, completely wetting both sides of the leaf. The three plants with the same inoculum line were enclosed in a plastic bag with a wet paper towel and left in the dark to incubate for 48 h. After this period, the plants were uncovered and grown randomized in a greenhouse, with bottom watering for 7 days. Several leaflets with necrotic symptoms were collected from each plant, surface sterilized for

1 min in 70% ethanol followed by 1 min in 0.5% sodium hypochlorite. Each leaflet was placed in a Petri dish with malt extract agar (2% malt extract, 1.5% agar). Tips of *Stemphylium* hyphae that grew out of the leaf pieces were transferred onto V8 agar and allowed to grow until sporulating, 10–14 days.

One isolate from each host lineage/fungal strain combination (24 isolates) was randomly selected (Generation 2). A spore suspension was prepared for each of these isolates, and used to inoculate three individuals of the same clover lineage from which it had just been isolated. *Stemphylium* was again isolated from symptomatic leaves from each of those clover/fungus combinations (Generation 3). This was repeated, with sequential inoculations and isolations to complete a separate serial passage for each of the original two *S. solani* strains through Generation 5. In each generation, the isolate was stored by freezing at –80°C in glycerol.

For the historical passage test, Generation 1 (the single spore isolates from *T. bifidum* and *T. barbigerum*) was inoculated onto 10 individuals of each of the 12 clover lineages. (Because of seed limitation, the European genotypes of *M. polymorpha* included only eight controls and eight replicates for the Generation 1 of the barb34L strain.) To examine the effects of serial passage through a single host, the Generation 5 isolates from each strain/lineage combination were inoculated onto 10 individuals of the clover lineage through which it had been passed. Density of spores in the inoculum was not different across generations (paired *t*-test, Barb34L lines: Gen1 =  $12.2 \pm 1.7$  vs. Gen5 =  $13.3 \pm 1.0$  thousand spores per mL,  $t_{10} = 1.8$ ,  $P = 0.097$ ; Bifi4C: gen1 =  $6.36 \pm 2.1$  vs. gen5 =  $7.3 \pm 1.4$  thousand spores per mL,  $t_{10} = 1.49$ ,  $P = 0.17$ ). For each clover lineage 10 water-dipped plants were included as controls. All these test inoculations were conducted in one large experiment, so that the Generation 1 inoculations serve both for the historical passage and serial passage analyses. Unfortunately, despite numerous attempts, we were unable to successfully infect and re-isolate *Stemphylium* from the European genotypes of *T. campestre*. As such, there are no tests of serial passage through that clover lineage, and it was only included in the Generation 1 historical passage inoculation. We did not conduct inoculations onto clover lineages other than those through which the fungi had been passed.

### Inoculation procedure

Plants were grown by nicking seed coats with a razor, placing the seeds on moist filter paper until germinated, and then transplanting the germinated seeds into Cone-tainers (2.5 × 16.5 cm; Stuewe and Sons, Corvallis, OR) filled with Premier Pro Mix HP potting soil (Premier

Horticulture, Red Hill, PA). Conetainers were randomized into racks and placed into a growth chamber (as above). Plants were grown in the growth chambers for approximately 5 weeks, until all plants had three or more true leaves. Rack positions in the growth chamber were changed regularly.

The three most recently expanded leaves on each plant were then marked by lightly tying red, white, or blue thread around the petiole; the colored threads allowed us to follow individual leaves of different ages.

We dip-inoculated each plant as above. Conetainers with the inoculated clovers were then placed inside a plastic bag, along with a wet paper towel to retain high humidity, and kept out of direct light for 48 h to allow for infection. Then plants were completely randomized in a greenhouse. Each Conetainer was inserted into an inverted paper cup resting inside a 10-cm saucer, to allow for bottom watering of the plants.

#### Evaluation of infectivity and virulence

For each of the three marked leaves, we followed symptom development and senescence at 3-day intervals for 4 weeks. Each leaflet was visually rated for severity of necrosis (scale 0%, 1%, 5%, 30%, 50%, 70%, 90%, 100% as used in Parker and Gilbert 2007). We surface sterilized and isolated fungi (as above) from each of the marked leaves at the time of senescence or at the conclusion of the experiment 4 weeks after inoculation. Each plate was examined under a stereoscope 1 week after isolation for the characteristic production of *Stemphylium* spores, and recorded as infected or not infected. Leaves did not have to be symptomatic to be infected. In total, we evaluated disease and infection on three leaves from each of 575 plants (1725 leaves).

#### Analyses

We had three different measures of the effect of the *Stemphylium* strains on each host lineage: infection rates, development of leaf necrosis, and time to leaf senescence. For each of these variables, we took both a serial passage approach and a historical opportunity approach to ask if the rates: (i) changed after four generations of serial passage, (ii) varied among clover lineages of different origins, where three clover lineages are nested within each of four origins (Native-CA, Exotic-CA genotype, Exotic-EU genotype Novel-EU), and (iii) differed between California and European genotypes (seed sources) of the European species introduced to California, paired by species.

All analyses were performed using JMP 7.0.2 (SAS Institute, Cary, NC).

#### Infection rates

For each plant we first calculated the proportion (of three) leaves that were successfully infected by *S. solani* after inoculation. (i) To examine whether serial passage through a host affected the probability of infecting that host, we used a paired *t*-test (Generation 5 vs. Generation 1 within each of the 11 host taxa) to test whether the overall change in infection rate was different from zero. This change is graphically presented as the difference (Generation 5 – Generation 1) in total proportion of leaves infected for each host species ( $D_{5-1}$  Proportion Leaves Infected). (ii) For the comparison across host types (Generation 1 only), we used a nested ANOVA, with plant species nested within origin, and each plant was a replicate. Where the full model and origin effect were statistically significant ( $\alpha = 0.05$ ), we compared means across origin using the Tukey HSD *post-hoc* comparison. We analyzed data from the two fungal strains separately. (iii) For the comparison of European and California genotypes, we calculated the mean proportion of leaves infected for each host-fungus combination (Generation 1 only) for each seed source of the three species (*M. polymorpha*, *T. campestre*, and *T. glomeratum*). We then compared infection rates of California and European genotypes using a paired *t*-test (CA vs. EU), with data from both fungal strains combined.

#### Development of necrosis

We used the area under the disease progress curve (AUDPC) as an integrated measure of disease severity on inoculated leaves. AUDPC was calculated using the trapezoidal method by determining the average disease rating for each temporally adjacent pair of measurements, multiplying that by the length of the time interval, and then summing across all intervals (Madden et al. 2007). To adjust for background necrosis unrelated to pathogen infection, we then calculated  $D_{1-C}$ AUDPC as the mean AUDPC of the three marked leaves on each plant minus the mean AUDPC for noninoculated control leaves for that host species. Positive values of  $D_{1-C}$ AUDPC indicate more necrosis after inoculation, and negative values indicate more necrosis on control plants. Statistical analysis of  $D_{1-C}$ AUDPC was the same as for infection rates, with (i) paired *t*-test to assess changes in AUDPC after serial passage, first calculating  $D_{5-1}$ AUDPC (Generation 5 – Generation 1), (ii) nested ANOVA, with species nested within origin, to test for differences among the plant origins, and (iii) paired *t*-test of the six host-fungus combinations to compare 'familiar' to 'unfamiliar' interactions.

#### Leaf senescence

Leaves were considered senesced if they fell from the plant or reached 100% necrosis. Observations were

labeled as right censored if leaves were still alive at the time of harvest (34 days). For the 30 leaves of each of the host-fungus combination (10 plants  $\times$  3 leaves per plant), we used Kaplan–Meier Survival Analysis to calculate the mean leaf survival time for that combination. Those species-wise mean leaf survival estimates were then used for analyses parallel to those described for necrosis, except without nesting, since there was but one estimate per species. In this case, negative values of  $D_{5-1}$ AUDPC indicated that leaves senesced more quickly when inoculated with Generation 5 strain than the corresponding Generation 1 strain, and negative values of  $D_{1-C}$  Leaf Survival Time indicated that leaves senesced that many days more quickly in inoculated plants than in control plants.

## Results

### Serial passage experiment

Four generations of serial passage of each of two strains of *S. solani* on each of 11 clover taxa (22 separate lineages) led to significant increases in infection rates, but there were no consistent changes in virulence (i.e., disease impacts on the host) (Fig. 2). In each case, we compare infection, necrosis, or senescence after inoculation with Generation 5 strains to that after inoculation with Generation 1 of the same lineage.

### Infection

For both strains of *S. solani*, four generations of serial passage caused significant and substantial overall increases in the ability of the fungus to infect leaves (Fig. 2). The change in infectivity was more than twice as much for lineages of the barb34L strain as for bifi4C.

### Development of necrosis

There was no directional change after serial passage for the effect of inoculation on necrosis (measured as AUDPC; barb34L:  $t_{10} = 0.28$ ,  $P = 0.78$ ; bifi4C:  $t_{10} = 0.10$ ,  $P = 0.92$ ). Some fungal lineages appeared to increase and others to decrease in virulence. Complementary analysis including only those leaves from which *S. solani* was isolated at the end of the experiment also showed no significant change in AUDPC (not shown).

### Leaf senescence

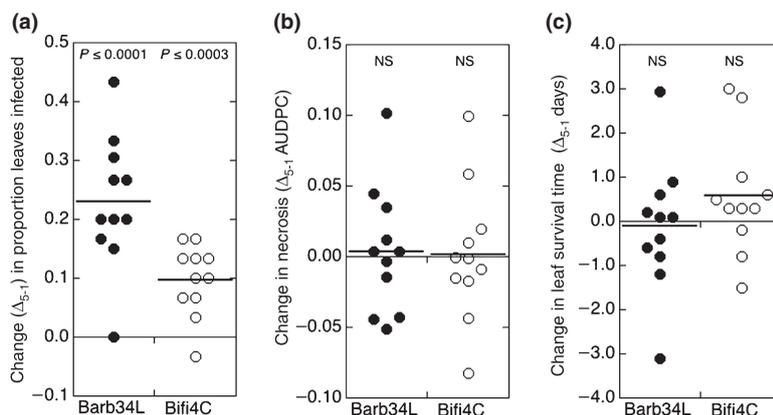
Consistent with the patterns for necrosis, there was no overall directional change after serial passage for the effect of inoculation on leaf survival (Fig. 2: barb34L:  $t_{10} = 0.26$ ,  $P = 0.80$ ; bifi4C:  $t_{10} = 1.41$ ,  $P = 0.19$ ). Some fungal lineages increased and others decreased in virulence.

### Historical opportunity analysis: difference across host origin

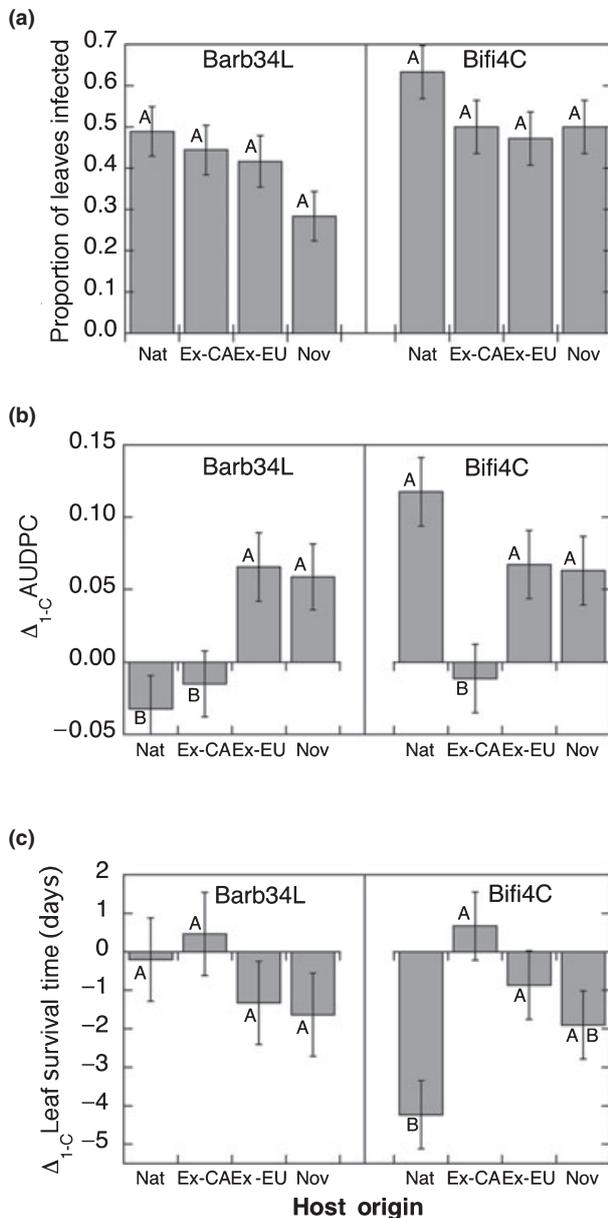
The effect of host origin (California Native, Exotic – California genotype, Exotic – European genotype, or Novel European species) on the impact of inoculation with two strains of the pathogen *S. solani* varied across the three different metrics of interaction: infection, necrosis, and leaf senescence (Fig. 3).

### Infection

The proportion of leaves successfully colonized after inoculation with *S. solani* (Generation 1) varied greatly among clover species for both fungal strains (ranging from 10% to 77% of leaves) but there were no significant differences in the proportion of leaves infected across host origin



**Figure 2** Effect of four generations of serial passage of strains barb34L and bifi4C of *Stemphylium solani* on each of 11 clover taxa. (a) The proportion of leaves infected after inoculation was significantly greater after four generations for both strains (barb34L:  $t_{10} = 6.76$ ,  $P = 0.0001$ ; bifi4C:  $t_{10} = 5.32$ ,  $P = 0.0003$ ). (b) There was no overall directional change for necrosis (measured as AUDPC; barb34L:  $t_{10} = 0.28$ ,  $P = 0.78$ ; bifi4C:  $t_{10} = 0.10$ ,  $P = 0.92$ ) or (c) leaf survival (barb34L:  $t_{10} = 0.26$ ,  $P = 0.80$ ; bifi4C:  $t_{10} = 1.41$ ,  $P = 0.19$ ), with some strains increasing and others decreasing in virulence.



**Figure 3** Effect of inoculation with two strains of *Stemphylium solani* on (a) proportion of leaves infected, (b) severity of leaf necrosis (as AUDPC, Area Under the Disease Progress Curve), and (c) mean leaf survival time, for three clover species from each of four origins: Native, Exotic-California genotype, Exotic-European genotype, and Novel European. For necrosis and survival time, values are shown relative to those for control plants. Means with the same letter (within a fungal strain/attribute) are not statistically different (Tukey's HSD test, alpha = 0.05); see text for statistical details. Error bars are standard errors from least-square means estimates.

(Fig. 3A) (barb34L: Model  $F_{11,106} = 2.35$ ,  $P \leq 0.013$ , effect test for origin  $F_{3,8} = 2.17$ ,  $P \leq 0.096$ ; bifi4C: Model  $F_{11,108} = 1.76$ ,  $P \leq 0.069$ , effect test for origin  $F_{3,8} = 1.26$ ,  $P \leq 0.291$ ).

#### Development of necrosis

Clover species of different origins differed significantly in the amount of necrosis caused following inoculation with *S. solani*, but the results were not completely consistent across the two fungal strains (Fig. 3B). For both strains, necrosis (measured as  $\Delta_{1-C}$ AUDPC, the difference in AUDPC after Generation 1 inoculation minus AUDPC of the control inoculations) was significantly greater on plants grown from European-collected seeds (both novel European species and European genotypes of established exotics) than in the California genotypes of the exotics. However, the barb34L strain caused significantly less damage, while bifi4c caused significantly more damage, on native clovers than on the exotics. (barb34L: Model  $F_{11,106} = 4.69$ ,  $P \leq 0.0001$ , effect test for origin  $F_{3,8} = 4.66$ ,  $P \leq 0.0042$ ; bifi4C: Model  $F_{11,108} = 3.179$ ,  $P \leq 0.001$ , effect test for origin  $F_{3,8} = 5.03$ ,  $P \leq 0.003$ ).

#### Leaf senescence

Leaf senescence is the most extreme manifestation of necrosis, and the patterns of senescence across host origin generally mirrored those observed for necrosis (Fig. 3C), although the differences were not statistically significant for the barb34L strain (Model  $F_{3,8} = 0.82$ ,  $P \leq 0.52$ ). Inoculation with the bifi4c strain tended to show the strongest reduction of leaf survival time on native clovers (Model  $F_{3,8} = 5.37$ ,  $P \leq 0.026$ ).

#### Historical opportunity analysis: 'familiar' versus 'unfamiliar' interactions

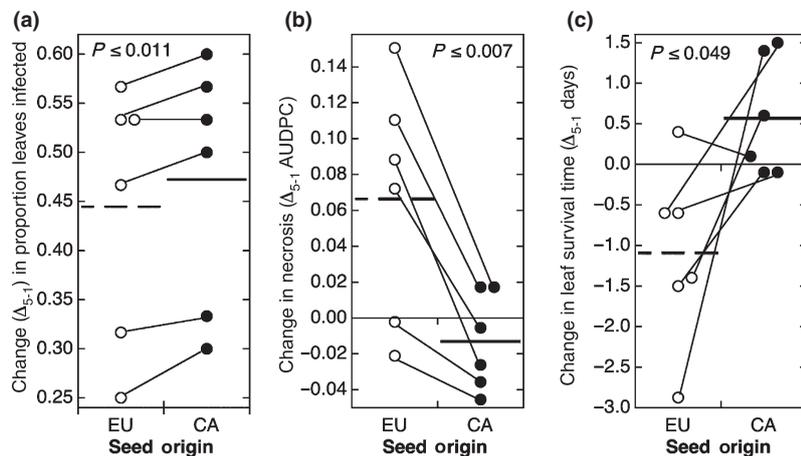
Substantial variation among taxa in morphology and physiology could obscure the effect of familiarity between the host and pathogen. We controlled for this problem in focused tests comparing introduced ('familiar') CA genotypes to novel ('unfamiliar') EU genotypes of the same species. There were marked differences in the impacts of *S. solani* on exotic clovers grown from seed collected in California compared to the same species grown from European seed (Fig. 4).

#### Infection

*Stemphylium solani* was more likely to infect Exotic clovers grown from California-collected seed than clovers of the same species grown from seeds collected in their native Europe (Fig. 4). The difference was moderate (6.6% greater on CA-genotypes), but consistent.

#### Development of necrosis

European genotypes of exotic clovers suffered consistently and significantly more necrosis after inoculation with *S. solani* than did California genotypes of the same species (Fig. 4). AUDPC in all six of the fungus-host



**Figure 4** Differences in the effects of inoculation with two strains of *Stemphylium solani* on European and California genotypes of three species of European clovers that have established as exotics in California. Each pair of circles connected by a line represents one of three clover species inoculated with one of two strains of *S. solani* (Generation 1). Thick horizontal bars indicate the mean for the six species-fungus combinations, and the *P*-values indicate whether the means are different between the seed origins by paired *t*-test. Three measure of plant-fungus interaction are the (a) proportion of leaves infected ( $t_5 = 3.95$ ,  $P = 0.011$ ), (b) severity of leaf necrosis (as AUDPC, Area Under the Disease Progress Curve) ( $t_5 = 4.47$ ,  $P = 0.007$ ), and (c) mean leaf survival time ( $t_5 = 2.59$ ,  $P = 0.049$ ). For necrosis and survival time, values are shown relative to those for control plants.

combinations decreased with familiarity, by up to a factor of seven. In two combinations, infection resulted in more necrosis than the control for the ‘unfamiliar’ interaction, but less necrosis than the control for the ‘familiar’ interaction. In other words, the familiar interaction was consistently less virulent, sometimes even becoming apparently beneficial to the host.

#### Leaf senescence

Inoculation with *S. solani* caused a reduction in leaf survival time on most of the European genotypes, whereas inoculated leaves of California genotypes tended to survive longer than did control leaves. As a result, inoculated leaves of the European genotypes had significantly shorter survival times than did California genotypes (Fig. 4).

#### Discussion

This experimental study of a generalist fungal pathogen across 12 hosts synthesizes information from a range of time scales on the potential for evolution to influence the disease dynamics of introduced species. Serial passage experiments demonstrated rapid evolution in the pathogen’s ability to infect a host, but found no change in the amount of damage caused. Comparative inoculation experiments did not provide strong support for the prediction that infection and virulence should be highest on native hosts and lowest on novel hosts. However, a directed comparison of California pathogens on local (California) genotypes versus novel (European)

genotypes of three introduced host species found greater infectivity and lower virulence on California genotypes, supporting the hypothesis of adaptive evolution in both host and pathogen over intermediate time scales.

#### Evolution of infection ability

Our serial passage experiment showed that *S. solani* is capable of rapidly evolving increased efficiency in the ability to infect a particular host species. Previous serial passage experiments with pathogenic fungi and oomycetes have focused primarily on changes in damage to the host, and seldom measure changes in the ability of the pathogen to infect the host *per se*. Those cases where increased ability to infect new host was documented directly have been limited to demonstrating selection against particular *avr* genes in the pathogen associated with specific host resistance genes, allowing infection of cultivars that were previously resistant (Martens 1973; Rutherford et al. 1985; Kolmer 1990). We are not aware of a previous serial passage experiment that specifically measured changes in infection ability independently from a measure of damage to the host.

We saw adaptive evolution of the pathogen despite a putatively strong genetic bottleneck caused by the use of a single spore isolate for each generation. However, Ascomycete fungi have various mechanisms that can maintain genetic variation. Each multicelled conidium (mitospore) of *Stemphylium* contains several nuclei. The nuclei within

individual conidia of many fungi (including in the closely related genus *Alternaria*) have been shown to derive from genetically distinct initial nuclei, and isolates from different germ-tube tips growing from a single conidium can vary in their pathogenicity (Stall 1958; Lee et al. 1984). Even with such genetic variability among nuclei within single-spore isolates, our estimates of the rate of adaptation in the pathogen are probably conservative relative to the potential for evolution in a more diverse pathogen population.

Many species in the anamorphic (asexual) genus *Stemphylium* have their teleomorphic (sexual) state in the genus *Pleospora* (Câmara et al. 2002). However, no sexual state has been reported for *S. solani*, and sexual reproduction is likely to be very rare. As for many other plant pathogenic fungi, genetic variation can be generated and maintained through a variety of asexual mechanisms (Pontecorvo 1956; Kistler and Miao 1992; Zolan 1995; Burdon and Silk 1997; Forgan et al. 2007). Although we have not found previous studies that measured variation in infectivity for *S. solani*, significant variation has been reported for host range, virulence, and toxin production (Mehta and Brogin 2000; Zheng et al. 2009).

Pathogen responses to serial passage varied substantially between the two pathogen strains. This variation could reflect different levels of available genetic variation or the presence of particular infection- or virulence-related alleles in the different fungal lineages, as these went through multiple generations of genetic bottlenecks along with selection. Similarly, pathogen responses varied across the clover hosts, which could reflect differences in genetic diversity at resistance loci or even differences in the nature of the traits involved in conferring resistance.

Serial passage experiments in agriculture have shown that there can be tradeoffs in the ability to infect multiple hosts, where an increase in virulence on a new host is accompanied by a loss of virulence on the original host (Ao and Griffiths 1976; Harrower 1977; Bardin and Leonard 2000). On the other hand, in some cases increased infection ability on one host can confer greater, rather than reduced, ability to infect a second host (Kolmer and Leonard 1986). We did not try to cross-inoculate our passaged fungi through alternative hosts, so we cannot evaluate this possibility in our system. However, such patterns of cross-infection could have important implications for the consequences of novel host invasions. In particular, tradeoffs could constrain the evolution of generalist pathogens on novel invaders that are at low density in the invaded habitat. On the other hand, if a novel host becomes very abundant so that the combination of increased familiarity and virulence tradeoffs led to the pathogen effectively 'switching' to the new host, the

impact on the native host population could actually be reduced.

We did not find clear patterns in the ability of the pathogen to infect different host types (native, introduced-familiar, introduced-unfamiliar, novel). While the trend was for greatest infection on native species, the dramatic variability among host species swamped any effect of origin. Many factors can influence the susceptibility of plants to pathogen infection, resulting in variation among host species such as we saw. In past work, we found that for *Stemphylium*, the retention of water on leaves (determined primarily by leaf size) was a key factor influencing plant disease development among clover species (Bradley et al. 2003). Our results suggest a strong potential for rapid evolution in infection ability as pathogens become more familiar with novel hosts, but the degree of change would be determined by a complex set of traits of both plant and pathogen.

We controlled for this variability among host species while investigating the effect of shared evolutionary history by comparing 'familiar' to 'unfamiliar' genotypes of clover species introduced from Europe to California. The introduction of a novel host species provides an opportunity to study the evolutionary outcomes of novel host-pathogen interactions. In our study, we found that consistently and significantly, the ability to infect the host was enhanced in the host-pathogen pair that shared an evolutionary history of at least several decades. Depending on the details of evolutionary dynamics, time since introduction could influence how different the 'familiar' interaction is from the corresponding 'unfamiliar' interaction. The three host species we used varied in their estimated time since introduction to California. Unfortunately, there is no record of exactly when each of these species reached BMR; however, we know that *M. polymorpha* was an early Spanish introduction (<1800), *T. campestre* was introduced by 1894, and *T. glomeratum* was first collected in California in 1927 (Parker and Gilbert 2007). However, all three host taxa show about the same increase in infection rates from 'unfamiliar' to 'familiar' genotypes.

Our study is limited by the fact that for logistical reasons, we have included only one pathogen species and three hosts, and compared those interactions between only one invaded site and one site from the native range. Our results are consistent with a pattern of evolution in the host since time of introduction; however, we cannot rule out alternative explanations having to do with site differences. All three introduced hosts showed similar patterns, across two fungal genotypes isolated from different species, which makes it unlikely that these are random changes. But if there were more disease pressure at our collection site in the native range than at BMR, there might be a greater resistance response to all pathogens in plants from that site.

A reciprocally designed experiment with a Mediterranean pathogen (which could not legally be done in California) could be used to test this alternative hypothesis.

### Evolution of virulence

If the need for horizontal transmission constrains the evolution of virulence in the wild (Herre 1993; Lipsitch et al. 1996), virulence should increase in a serial passage experiment, where transmission is assured. However, after serial passage through eleven clover species, virulence of *S. solani* did not change in a consistent, directional way. Neither the development of necrosis on leaves (quantified as AU-DPC) nor the speed of leaf senescence increased as predicted, even though infection rates increased significantly. There are several possible reasons for this result. First, the evolution of virulence could have been constrained by the limited genetic variation in the pathogen, as described above, reducing the available variation in virulence-associated genes. Second, it is possible that evolution of the pathogen was constrained by unintentional selection for traits important in some aspect of the laboratory procedure (e.g., growth and ability to sporulate on agar media in the lab), if those traits were antagonistic to traits important in causing necrosis on leaf tissue.

Third, the lack of change in virulence in the face of substantial change in infection rates could be explained by the specifics of the biology of *S. solani*. *Stemphylium* is a necrotrophic pathogen that infects leaves, then produces non-host-specific compounds that kill plant tissue, which it can then colonize and use for reproduction (Mehta and Brogin 2000; Zheng et al. 2009). Application of purified culture filtrate of the pathogen causes host necrosis (Zheng et al. 2009). Therefore, the fungal traits important in infection are separate from those important in the production of necrosis. In this case, sequential generations of inoculation and re-isolation in a serial passage experiment should lead to selection for strains with increased infection efficiency, without regard to how much necrosis was produced. Many serial passage experiments in the literature that show consistent increases in virulence have been conducted with obligate biotrophs, where the ability to successfully infect and reproduce on the host is the primary measure of virulence (e.g., Groth and Roelfs 1982), or where the necrotrophic pathogen produces host-specific toxins required to cause disease (e.g., Harrower 1977). Serial passage with polyphagous necrotrophs (as is *Stemphylium*) does not consistently show increases in virulence (e.g., Rozycki et al. 1990). This underscores the importance of specific traits in the pathogen and the plant in determining the direction and rate of evolutionary change as novel pathogen-host combinations become increasingly familiar.

Comparing virulence across the four types of host resulted in complex patterns. The Bifi4C strain was strongly pathogenic on native clover hosts, having an impact on these species that was about twice as strong as for any other combination. One native species in particular (*T. macrei*) was extremely sensitive to infection with Bifi4C. With the exception of this very strong interaction, the more familiar host-pathogen combinations (i.e., native and CA genotypes of introduced clovers) were less virulent, showing slower disease progress and leaf senescence, than in the more novel combinations.

Interestingly, this apparent loss of virulence in more familiar interactions was dramatic and consistent in the pairwise comparisons of EU and CA genotypes of introduced clovers. While the pathogen was better at infecting 'familiar' hosts, it caused less damage on them, rather than more. Classic models that predict an evolutionary loss of virulence depend on the assumption that between-host transmission is negatively correlated with within-host virulence (May and Anderson 1983; Frank 1996). We have little reason to expect transmission to be negatively correlated with leaf senescence for *Stemphylium*, which sporulates from senescent leaf material. However, it is possible that by persisting in healthy leaves that stay on the plant longer, the fungus could benefit from increased opportunities to infect new leaves as they develop on the plant.

We suspect that the pattern of reduced virulence reflects an evolutionary adjustment on the part of the plants in response to a ubiquitous aspect of their new biotic environment. *Stemphylium* infects a very high proportion of plants at BMR (Parker and Gilbert 2007). We have demonstrated in fungicide experiments that the interaction with foliar fungi at BMR is clearly pathogenic in some years, but can be mutualistic in other years (Parker and Gilbert 2007). That is, plants actually benefit from having fungi in their leaves in some years, possibly related to particularly dry conditions. In such a system, plants could evolve mechanisms that increase their tolerance of infection, or that tip the consequences of infection from negative to positive. Interestingly, consistent with this scenario, the impact of fungal infection actually went from negative to positive in some of our comparisons of EU genotypes to CA genotypes. That is, leaves of infected plants were dropped more quickly than control leaves when the host was unfamiliar with the fungus, while infected leaves were retained longer than controls when the host and fungus shared a history.

### Implications for biological invasions

While interactions between introduced species and their natural enemies have taken center stage in explanations of biological invasions, little attention has been paid to how

evolution could influence these biotic interactions. In our system, a host-generalist fungus is by far the most common foliar pathogen, and evolution of this species to exploit the available hosts, both native and introduced, may involve a subtle process of adjusting to individual host species depending on their relative densities and other factors. We have shown through serial passage experiments that a pathogen can evolve rapidly in its ability to infect novel hosts, and we found patterns across hosts consistent with increased infection in more familiar interactions. This suggests that, in cases where introduced species experience an advantage through release from natural enemies, there is strong potential for evolution to contribute to the reduction of that advantage over time.

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