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Local Biotic Environment Shapes the Spatial Scale of Bacteriophage Adaptation to Bacteria

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ABSTRACT: The ecological, epidemiological, and evolutionary consequences of host-parasite interactions are critically shaped by the spatial scale at which parasites adapt to hosts. The scale of interaction between hyperparasites and their parasites is likely to be influenced by the host of the parasite and potentially likely to differ among within-host environments. Here we examine the scale at which bacteriophages adapt to their host bacteria by studying natural isolates from the surface or interior of horse chestnut leaves. We find that phages are more infective to bacteria from the same tree relative to those from other trees but do not differ in infectivity to bacteria from different leaves within the same tree. The results suggest that phages target common bacterial species, including an important plant pathogen, within plant host tissues; this result has important implications for therapeutic phage epidemiology. Furthermore, we show that phages from the leaf interior are more infective to their local hosts than phages from the leaf surface are to theirs, suggesting either increased resistance of bacteria on the leaf surface or increased phage adaptation within the leaf. These results highlight that biotic environment can play a key role in shaping the spatial scale of parasite adaptation and influencing the outcome of coevolutionary interactions.

Keywords: coevolution, plant pathogen, local adaptation, geographic mosaic, hyperparasite, community composition, parasite-mediated selection.

Introduction

The spatial scale at which parasites adapt to their hosts can play a key role in structuring communities (Rodriguez-Brito et al. 2010) and populations (Haldane 1949; Buckling and Rainey 2002*a*), influencing species invasions (Tompkins et al. 2003; Prenter et al. 2004), and determining the epidemiological consequences of gene flow (Lively 1999; Gandon 2002; Morgan et al. 2005). It is well established that interactions across multiple trophic levels can significantly alter coevolution between hosts and parasites (a type of G × G × E interaction, where the third species can be viewed as the biotic environment in this context; e.g., Clancy and Price 1986; Mopper et al. 1995; Taylor et al. 1998; Thompson 1999, 2005; Cory and Myers 2004; Craig et al. 2007; de Roode et al. 2008). Accordingly, the distribution of other interacting species (and spatial heterogeneity in general; Gandon and Nuismer 2009) is likely to play a large role in shaping the spatial scale of parasite adaptation (or maladaptation) to hosts. A simple prediction is that parasite (or host) local adaptation (i.e., performance against local relative to foreign hosts [or parasites]) will be more pronounced where host-parasite communities are sampled across environments with high variation in terms of the other interacting species. This is because different resistance (or infectivity) traits are likely to dominate in different localities through direct or correlated selection imposed by the other interacting species (Laine and Tellier 2008). Host-parasite interactions between bacteria and viruses living in much larger organisms provide a system to test this prediction, because local adaptation can be measured both within the larger host organism and among hosts, that is, under conditions of low (within the host) and high (among hosts) biotic variance. Here, we examine phage local adaptation to natural bacterial populations collected from either the surface or the interior of leaves from horse chestnut trees (*Aesculus hippocastanum*).

Viruses of bacteria (bacteriophages) are ubiquitous and play a key role in regulating bacterial communities (Bohannan and Lenski 2000). Many viruses require cell lysis to complete their life cycle, resulting in strong selection for bacterial host resistance and in turn for phage infectivity, at least under laboratory conditions (Buckling and Rainey 2002*a*; Forde et al. 2004; Morgan et al. 2005; Brockhurst et al. 2006; Lopez-Pascua and Buckling 2008). These coevolutionary interactions can readily lead to local ad-

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aptation of phages under a range of controlled conditions. Furthermore, recent studies have detected phage adaptation in natural soil (Vos et al. 2009) and hot-spring (Held and Whitaker 2009) populations, but how this local adaptation changes with scale has yet to be investigated. Plant-associated bacteria are ideal for investigating questions of scale because of their natural "patchy" spatial distribution (Parker 1991; White and Gilligan 1998). Moreover, a wide range of plant-associated bacterial species are pathogenic, so that understanding the scale of phage adaptation in these plants has important implications for disease epidemiology and the use of therapeutic phages. In the case of horse chestnut trees, *Pseudomonas syringae* are particularly important bacterial pathogens (Webber et al. 2008), infecting an estimated 70%–80% of the trees in Oxfordshire, according to the United Kingdom Forestry Commission.

We expected an increase in local adaptation of phages to plant-associated bacteria with increasing spatial scale for two reasons: first, large distances should limit both phage and bacteria gene flow. This effect should be largely independent of bacteria-plant interactions and should operate similarly for free-living and parasitic bacteria. Second, bacterial communities are likely to differ among plant hosts (and thus space) because key bacterial adaptations are typically required to live in or on host plants (Morris and Monier 2003; Sabaratnam and Beattie 2003; Melotto et al. 2006; Kniskern et al. 2007), and these adaptations are likely to be specific to the plant genotype and species with which the bacteria is interacting (e.g., Wilkinson et al. 1996; Guttman et al. 2002; Sarkar et al. 2006). Teasing apart these mechanisms is difficult in natural populations, but in an attempt to assess their importance, we sampled bacteria and phages from two areas of the plant that are in close proximity but likely differ in terms of the strength of plant interaction with bacteria: the surface and the inside of leaves. Because aerial dispersal of bacteria is now known to be a common occurrence, surface populations of bacteria will be subject to constant immigration (Lindow and Brandl 2003; Christner et al. 2008; Morris et al. 2008), and the bacteria-phage interactions in these environments are likely to be shaped primarily by the scale of dispersal rather than specific bacteria-plant interactions. In contrast, bacterial populations within the more sheltered leaf-interior sites should be somewhat less transient and more specialized in their ability to evade plant immune response, allowing for persistent coevolutionary interactions with phages. We therefore predict greater phage local adaptation to bacteria within leaves, relative to that on the leaf surface.

Material and Methods

Collection of Bacterial Isolates and Phage Inocula

We examined the scale of interactions between naturally occurring phages and bacterial communities in the phyllosphere by collecting two leaflets (of which there are five to seven per leaf) from each of two leaves from a total of eight horse chestnut trees in Oxfordshire, United Kingdom, in October of 2008. We sampled bacteria (24 isolates per leaflet) and phages (one inoculum per leaflet) from both the leaflet surface and the leaflet interior (fig. 1) by agitating individual leaflet sections in 10 mL of 0.1 M potassium phosphate (pH 7.2) for 5 min and then removing the leaf and freezing the buffer with added glycerol at −20°C. Bacteria and phages from the leaf interior were then isolated from the same leaflets by surface sterilizing via submersion in a 0.02% Tween 20 and 1% sodium hypochlorite solution (Hirano and Upper 2000). After sterilization, leaflets were rinsed three times with sterile water, and three 2-cm sections of each sterilized leaflet were placed in buffer with four sterile 0.25-inch ceramic spheres. These sections were homogenized with a FastPrep-24 instrument (MP Biomedicals) and frozen with glycerol at −20°C. We selected 24 bacterial isolates per leaflet sample by thawing buffer solutions (containing either the presterilized leaf runoff [surface] or poststerilization homogenate [interior]), diluting them 1 : 100 in sterile buffer, and plating them on 1.2% Kings Broth (KB) agar plates for 24 h at 28°C. Bacterial isolates were chosen by randomly assigning a point on each plate and selecting the 24 colonies that were closest to the point, regardless of size or color. The remaining buffer (8 mL) was used to generate each of 64 phage inocula (one inoculum per leaflet from the surface and one inoculum per leaflet from the interior). This was done by adding 800 μL of chloroform to the buffer and centrifuging for 3 min at 13,000 rpm. Supernatants were stored in the dark at 4°C for use as inoculum during infection assays.

Identification of Species and Calculation of Species Diversity

We first tested the prediction that the composition of culturable bacterial species would be different across the surface and the interior of leaves despite the physical proximity of the microenvironments (Sabaratnam and Beattie 2003; Morris et al. 2008). In particular, we expected to find isolates representing the virulent pathogen *Pseudomonas syringae* pathovar *aesculi* (the causal agent of bleeding-canker disease in horse chestnut) within the leaf interior, as up to 80% of trees are currently infected in parts of Europe (Webber et al. 2008). Importantly, *P. syringae* has been shown to infiltrate the inner regions of the leaf

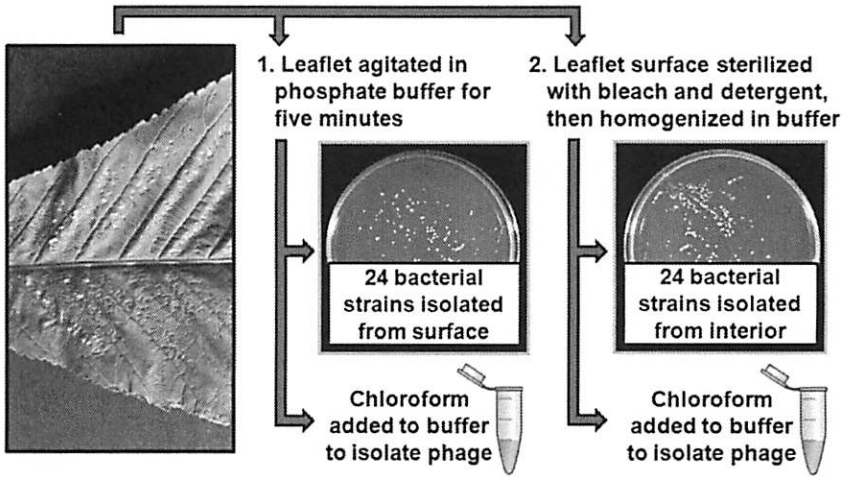

Figure 1: Sampling methods for bacteria and phage populations isolated first from the surface of leaflets and then from surface-sterilized and homogenized leaflets.

by overriding the plant's innate immune response and inducing the reopening of stomata (pores found on the leaf surface; Melotto et al. 2006).

We sequenced ~800 bp of the 16S ribosomal RNA region from 384 individual isolates (24 surface and 24 interior isolates from each of the eight trees examined). This analysis was restricted to culturable bacterial species, which is known to result in a significant underestimate of species diversity in microbial communities (Whipps et al. 2008). However, the pseudomonad bacterial species that most interested us from an epidemiological perspective are known to be culturable on KB agar (Webber 2008). Before sequencing, bacterial isolates were grown in KB overnight. q3 These overnight cultures were then diluted 1 : 10 in buffer and used as a polymerase chain reaction (PCR) template in reactions with the universal 16S primers 27f (Lane 1991) and 907r (Muyzer 1998). Diluted PCR products (1 : 10 in buffer) were sequenced with the 907r primer and then aligned and compared against the NCBI database with Geneious (ver. 2.5) software. Individual isolates were assigned to a given genus and species when possible according to the top Basic Local Alignment Search Tool hits q4 associated with the sequence (with an *e*-score of 0.0).

Sympatric diversity was calculated via the species-specific characterization, where possible, as the complement of Simpson's index (i.e., the probability that two randomly selected individuals are different species; Simpson 1949):

$$D = \left(1 - \sum_{i}^{s} p_i^2\right)\left(\frac{N}{N-1}\right),$$

where p_i represents the proportion of the ith species, N is the total number of isolates, and s is the total number of species. Allopatric diversity was calculated as a variance (Lande 1996) and is the probability that two isolates across populations are of different species:

$$d^2 = \sum_{j} q_j \sum_{i} (p_{ij} - \bar{p}_i)^2,$$

where $\bar{p}_i$ represents the mean proportion of the ith species across all j populations and q is a weighting factor for each population (equal to 1, since all populations had equal sample size). For all species-specific phage susceptibility analyses, isolates were grouped according to genus, as we could characterize all isolates to the genus level. However, the 49 isolates that could be clearly characterized as *P. syringae* (i.e., that had >99% sequence similarity to known *P. syringae* isolates) were considered separately, as this was the bacterium of primary interest.

Cross-Inoculation Experiments

We determined whether phage populations were adapted to local bacterial isolates by performing two separate reciprocal, cross-inoculation experiments (one for leaf-surface bacteria and phages and one for leaf-interior bacteria and phages). For each experiment, all 32 phage inocula (one per leaflet) were spotted, in a grid, onto a lawn of each bacterial isolate examined. Twenty (randomly chosen out of the possible 24) bacterial isolates from the surface and 20 from the interior of each leaflet were used in the

cross-inoculations, for a total of 1,280 bacterial isolates. Infection assays were performed with a standard agar overlay in which a given isolate was mixed into soft agar, poured into a square petri dish, and spotted with 8 μL of each of the 32 phage inocula (surface phages onto surface bacteria and interior phages onto interior bacteria) in a grid. Bacterial lawns were grown overnight at 24°C, which we found to be the best temperature for growth in soft agar, and each of the 1,280 bacterial isolates was scored for plaque formation (i.e., localized absence of bacterial growth due to phage infection and spread) within each phage inoculum spot (Vos et al. 2009). Phage presence in each inoculum was subsequently confirmed by subsampling of plaques from each leaflet and passaging, at various concentrations, through known lab strains of *P. syringae* to isolate individual phage clones.

We also examined the presence/absence of *Pseudomonas*-specific phages across microenvironments, using a common-garden experiment in which we infected 24 lab isolates (across six pathovars, isolated from 12 different host plant species) of *P. syringae* with each of the 64 inocula from the surface and interior of leaflets (strain information is provided in table A1 in the online edition of the *American Naturalist*). These characterized isolates were chosen because our primary interest was in *P. syringae*–specific phages and the design allowed for an independent assessment of infectivity of phages collected from the two microenvironments on *P. syringae* that have not been under recent selection for phage resistance. The total number of isolates infected by each inoculum was compared across the surface and interior inocula with an independent-samples *t*-test.

Statistical Analyses

We scored each of the bacterial isolates as either susceptible or resistant to each of the 32 phage inocula from the same microenvironment (i.e., surface phages on surface bacterial isolates and interior phages on interior bacterial isolates). We then calculated, for each phage inoculum, how many of the 20 isolates from each leaflet were susceptible. To examine overall differences in phage infection rates on local bacteria between the surface and the interior of each leaflet, we used a paired-samples *t*-test. This measure was used to estimate the density of infective phages in the microenvironment, but it is not indicative of actual phage particle numbers because (1) only phages that are targeting culturable species of bacteria can be quantified (Yang et al. 2001) and (2) only phages that are successful at infecting these sampled bacterial hosts can be quantified.

Next, we compared phage local adaptation between the surface and interior microenvironments, using a two-way ANOVA in which we rank-transformed the proportion of isolates infected to help correct for the unequal variances between the microenvironments (Conover and Iman 1981) and compared across sympatric (same leaflet) and allopatric (different leaflet) combinations. For subsequent analyses, we examined the two reciprocal cross-inoculations (surface and interior) separately. The scale of phage local adaptation was examined with a nested analysis in which the proportion of bacteria infected per leaflet was arcsine-square-root transformed. The leaflets from which each phage inoculum/bacterial population was derived (two per leaf, four per tree) were treated as replicates within a given leaf (LEAF), which was treated as a random effect, and each leaf was nested within a given tree (TREE), which was also treated as a random effect (analyses where TREE was treated as a fixed effect were also run and gave qualitatively similar results). Where we found significant bacteria × phage interactions, we tested specific hypotheses of local adaptation with orthogonal contrasts that compare infection success of sympatric versus allopatric phage-bacteria combinations at each scale (for similar analyses, see Thrall et al. 2002). We made this comparison by setting up two separate one-way ANOVAs (one for between-leaf comparisons and one for among-tree comparisons) and then performing contrasts within each analysis. For between-leaf contrasts, the mean proportion of bacteria infected by phages from sympatric leaves was compared with the mean from allopatric leaves within the same tree. For among-tree contrasts, data were aggregated within tree, and the average proportion infected for sympatric combinations was compared with the average proportion infected for allopatric combinations. Where the overall local-adaptation term was significant, we followed up with a series of specific local-adaptation contrasts for each tree (i.e., comparing phage infection on sympatric bacteria from its own tree with that on bacteria from all other allopatric trees). This analysis allowed us to determine both the overall strength of local adaptation and the consistency of the pattern across tree populations. We controlled for multiple comparisons of these contrasts by using the Dunn-Sidak method (Sokal and Rohlf 1995).

Because these latter comparisons employ the "home-versus-away" definition of local adaptation (Kawecki and Ebert 2004), we further examined the pattern of phage adaptation by using a reciprocal pairwise method that simultaneously compares the difference between sympatric and allopatric combinations (Vos et al. 2009) as "home versus away" and "local versus foreign." For this analysis, local adaptation was calculated for each leaflet pair as the mean difference ($S - A$) in the proportion of sympatric (S) and allopatric (A) bacterial isolates infected by the two phage populations. Each average $S - A$ can then be grouped according to the question of interest. First, pairwise combinations were categorized as being from either

same leaflet or different leaflets within a tree (i.e., all combinations across trees were left out), and the mean $S - A$ across all combinations was examined. Next, the mean proportions of bacteria from a given tree (across all leaflets combined) infected by phages from the same tree (S) and from different trees (A) were calculated, and the mean $S - A$ for each pairwise tree combination was determined. For these analyses, a measure of 0 indicates no phage local adaptation, a negative value indicates phage local maladaptation (equivalent to host local adaptation), and a positive value indicates phage local adaptation. This method also allowed us to examine phage adaptation to specific bacterial groups by choosing only pairwise interactions involving the host bacterium of interest.

Finally, we tested the importance of physical distance between trees on phage local adaptation by performing an ANCOVA, with linear distance as a covariate, phage source tree as a class variable, and mean proportion infected as the dependent variable. The dependent variable was arcsine-square-root transformed, and the covariate, distance, was log transformed. All analyses and figures were produced with PASW Statistics 18 (SPSS).

Results

Species Composition and Diversity between Microenvironments

Of the 384 isolates sequenced, 196 were found to be most similar to *Erwinia rhapontici*, 46 were most similar to *Erwinia billingiae*, 61 were most similar to *Pseudomonas* (across nine different species, not including *Pseudomonas syringae*), 49 were most similar to *P. syringae*, 21 were most similar to *Rahnella aquatilis*, and 11 were most similar to *Pantoea agglomerans*. The interior and surface microenvironments differed in dominant species composition (fig. 2*A*) and within-tree species diversity, such that the interior was significantly more diverse than the surface (interior: mean $D = 0.564$, SD = 0.14; surface: mean $D = 0.086$, SD = 0.15; paired-samples t-test: $t_7 = -5.93$, $P < .001$). Whereas the surface samples were composed mainly of *Erwinia*-like enterobacteria (a group that includes plant pathogens known to colonize crop plants and trees; Huang et al. 2007), the interior samples included both enterobacteria and pseudomonads. As predicted, more isolates of *P. syringae* were found within the leaf than on the leaf surface (Melotto et al. 2006; fig. 2*A*).

Furthermore, the surface samples had a much lower between-tree allopatric diversity than did the interior samples (surface: $d_2 = 0.0805$; interior: $d_2 = 2.0778$; $F_{7,7} = 25.82$, $P = .0002$), suggesting a greater influence of dispersal between trees on the leaf surface than in the leaf interior. The higher allopatric diversity of leaf-interior populations could also be the result of increased divergent selection, driven by increased tree host–mediated and/or phage-mediated selection on bacterial populations within the leaf relative to the leaf surface. This interpretation would be in accord with previous lab findings in which experimental passaging of phages was shown to increase diversity between replicate bacterial populations (Buckling and Rainey 2002*b*).

Bacterial genera in the leaf interior differed in their overall susceptibility to phages (fig. 2*B*; Kruskal-Wallis test: $\chi^2_3 = 31.39$, $P < .001$). We were unable to conduct a similar analysis for bacteria on the leaf surface because the surface populations were strongly dominated by *Erwinia*-like isolates and had overall low levels of susceptibility to phages. In the leaf interior, *Pseudomonas* spp. and *Erwinia* spp. were significantly more susceptible than all *Pantoea* spp. and *Rahnella* spp. (Mann-Whitney tests with sequential Bonferroni correction), indicating either that phages within the leaf are targeting the more common bacterial species or that *Pantoea* and *Rahnella* species are more likely to be resistant to phages. There was no significant difference between the *P. syringae*–like isolates and other pseudomonads ($P = .502$), but there was a marginally significant difference (given the Bonferroni-corrected cutoff P value of .017) between *Erwinia* spp. and *Pseudomonas* spp. isolates ($P = .028$). Finally, the results of the common-garden experiment in which all phage inocula were used to infect each of 24 lab isolates of *P. syringae* indicate that the leaf surface had fewer *P. syringae*–specific phages than the leaf interior (fig. 2*C*; fig. A1 in the online edition of the *American Naturalist*; independent samples t-test, equal variances not assumed: $t_{324} = -5.97$, $P < .0001$), as predicted by the lower frequency of pseudomonad isolates found on the leaf surface. Together, the results from our sequenced isolates show that bacterial community composition differed between the interior and the surface of leaves (fig. 2*A*) and that phages within the leaf were more infective to common bacterial genera (fig. 2*B*) and to previously characterized *P. syringae* isolates (fig. 2*C*).

Phage Local Adaptation across Microenvironments

With regard to phage infectivity to natural bacterial communities, we found that 45% of bacterial isolates (288 of 640) from the leaf interior were susceptible to at least one phage inoculum from the leaf interior. This was in stark contrast to the bacteria from the leaf surface, of which only 3% (21 of 640) were susceptible to phages from the leaf surface (paired-samples t-test: overall susceptibility: $t_{31} = -7.117$, $P < .0001$; susceptibility to sympatric phages [data not shown]: $t_{31} = -6.033$, $P < .0001$). As would be predicted from this disparate result between the two mi-

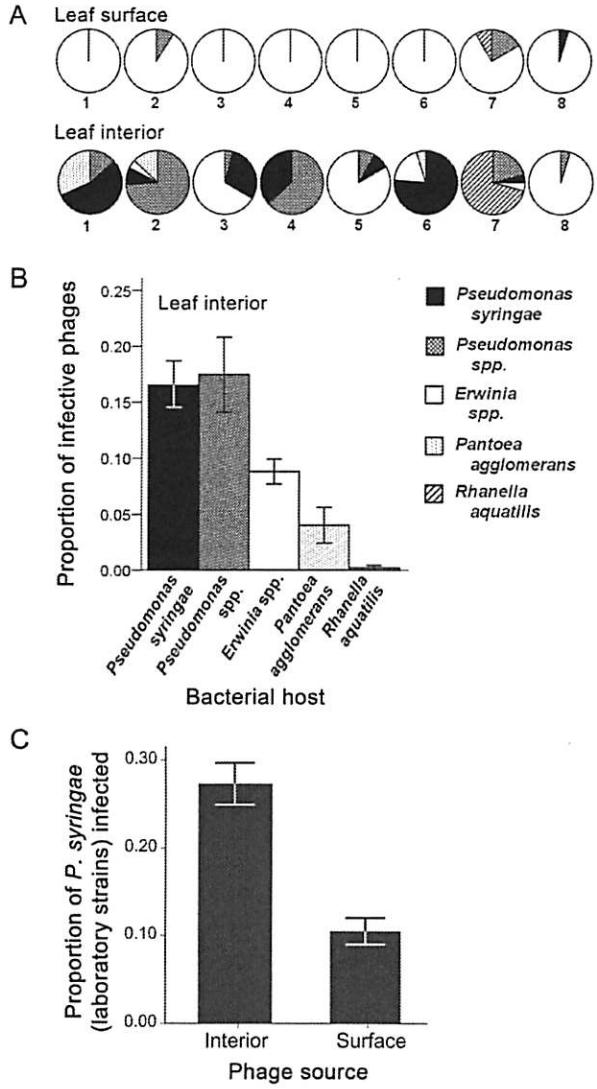

Figure 2: Species composition across microenvironments. *A*, Comparison of species composition across leaflet surface and interior for each of eight trees examined (proportion of each species out of 24 total isolates per microenvironment per leaflet). *B*, Mean susceptibility to phages (±SEM) across all sequenced isolates. Susceptibility was calculated for each isolate as the proportion of phage inocula (out of 32) that were infective. *C*, Infectivity of bacteriophages from interior and surface microenvironments on 24 lab isolates of *Pseudomonas syringae* (across nine pathovar types), calculated as the mean proportion (±SEM) of strains that were susceptible to phage inocula from the interior and the surface of each leaflet.

croenvironments, we found a stronger signature of local adaptation (greater infectivity to sympatric vs. allopatric bacteria) for phages from the interior than for those from the surface (fig. 3*A*; two-way ANOVA: main effect of microenvironment: $F_{1,204} = 100.33$, $P < .000$; sympatry: $F_{1,204} = 33.469$, $P < .000$; and interaction between microenvironment and sympatry, $F_{1,204} = 19.185$, $P < .000$).

Local adaptation of phages was also apparent for each of the two potentially pathogenic groups of bacteria, *P. syringae*–like and *Erwinia* species. Phages from the leaf interior were strongly adapted to sympatric *P. syringae*–like isolates, relative to allopatric isolates from different trees (fig. 3*B*; $t_{14} = 4.31$, $P = .0007$). Furthermore, we were able to compare susceptibility of *Erwinia*-like isolates

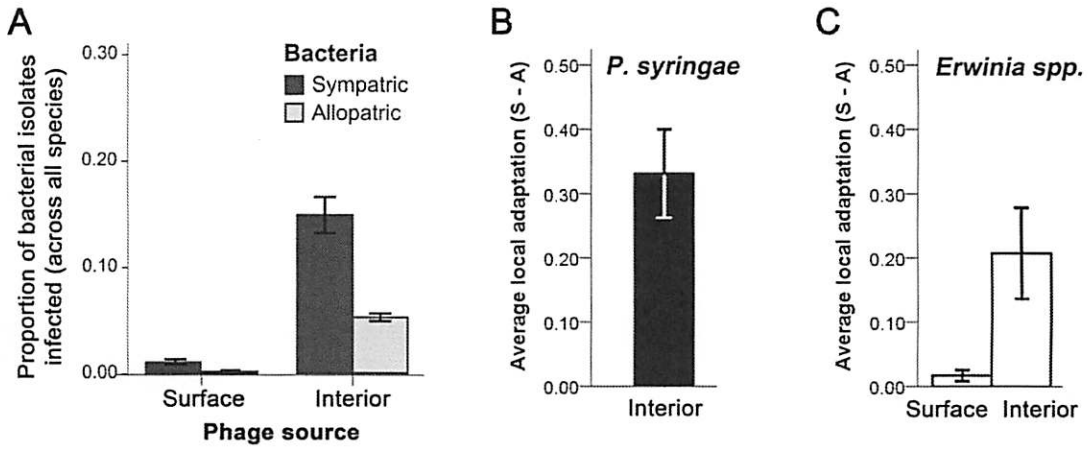

Figure 3: Local adaptation of bacteriophages. *A*, Mean phage local adaptation (±SEM) to *Pseudomonas syringae*–like isolates (from trees 1–6) from the leaf interior (data from the surface were excluded because of the low numbers of *P. syringae*–like isolates). *B*, Mean phage local adaptation (±SEM) to *Erwinia*-like isolates (from trees 3, 5, 6, and 8) from either the interior or the surface microenvironment. *C*, Mean proportion (±SEM) of all bacterial isolates from each leaflet susceptible to sympatric (generated from the same leaflet) versus allopatric phage inocula across the surface and interior. Proportion susceptible (out of 20 bacterial isolates per leaflet) was calculated for each of the 32 leaflets examined (four leaflets per tree across eight trees).

from the surface and the interior of leaves from four of the eight trees (those that had >15% *Erwinia*-like isolates) and, as predicted and in line with the results across genera, we found that the signature of local adaptation was greater within the leaf than on the leaf surface (fig. 3*C*; $t_5 = 2.81$, $P = .037$).

Although both microenvironments showed significant local adaptation of phage overall at the between-tree level (tables 1, 2; fig. 3*A*), phage local adaptation among surface populations was observed for only three of the eight trees (fig. 4; trees 2, 7, and 8 had significant phage local adaptation; $P < .01$, after multiple contrasts were controlled for), in contrast to the leaflet-interior populations, where significant local adaptation was observed for seven of the eight trees (fig. 4; phages from all trees but tree 4 were found to be significantly locally adapted to bacteria; $P < .01$, after multiple contrasts were controlled for). This result could be driven by either an overall lower phage prevalence or an overall higher level of bacterial resistance on the surface versus the interior of leaves. Either of these processes would reduce the statistical signature of local adaptation for the few phages that are able to infect local bacteria on the leaf surface.

Spatial Scale of Phage Local Adaptation

For both the surface and the interior cross-inoculations, phage local adaptation was apparent among trees but not among leaves within a tree (tables 1, 2; fig. 4). This result was largely confirmed by the pairwise analyses, where the mean $S - A$ (±SD) for surface bacteria-phage combinations from different leaflets within a tree was 0.007 ± 0.017 (one-sample *t*-test: $t_{47} = 2.801$, $P = .007$; note that, for this analysis, phage local adaptation at the within-tree scale is significant, although very small in effect) and that for surface bacteria-phage combinations from different trees was 0.010 ± 0.011 ($t_{27} = 4.818$, $P < .001$). Similarly, for the leaf interior, the mean $S - A$ for bacteria-phage combinations from different leaflets within a tree was 0.027 ± 0.316 ($t_{47} = 1.407$, $P = .166$), and for combinations from different trees it was 0.097 ± 0.035 ($t_{27} = 14.75$, $P < .001$). We found no effect of physical distance between trees on whether phages were successful at infecting allopatric bacterial communities (surface: $F_{1,887} = 0.238$, $P = .626$; interior: $F_{1,887} = 0.041$, $P = .840$).

Discussion

Using natural populations of bacteria and phages isolated from either the interior or the surface of horse chestnut leaves, we show that phages are strongly and consistently adapted to local bacterial populations. The data indicate that, across both the interior and the surface of leaves, the spatial scale of local adaptation is the host tree; phages are more likely to infect bacteria from the same tree but are not necessarily better at infecting bacteria from the same leaf within a given tree (fig. 4). Lack of local adaptation within trees is consistent with recent work showing that

Table 1: Variation in the proportion of surface bacterial isolates (out of 20 tested per leaflet) that were susceptible to phage inocula from the leaf surface in relation to their tree of origin and leaf of origin (nested within tree)

Source	df	MS	F	Partial η^2
Bacteria tree	7	.001	1.00	.50
Bacteria tree error	7	.001		
Phage tree	7	.001	13.24***	.92
Phage tree error	8	.001		
Bacteria tree × phage tree	49	.001	7.27****	.28
Local adaptation across trees	1	.010	66.04****	.58
Bacteria tree × phage tree error	929	.001		
Bacteria leaf within tree	7	.001	8.27****	.06
Bacteria leaf within tree error	929	.001		
Phage leaf within tree	8	.001	.28	.13
Phage leaf within tree error	15	.001		
Bacteria leaf × phage leaf within tree	15	.001	2.07*	.03
Local adaptation across leaves within tree	1	.008	1.73	.02
Bacteria leaf × phage leaf within tree error	929	.001		

Note: All effects were tested under the assumption that bacteria and phage leaf and tree terms were random (results are qualitatively equivalent when tree is treated as a fixed effect). The dependent variable, proportion infected, was arcsine-square-root transformed. When mean squared values were combined to achieve the appropriate error term, the Satterthwaite approximation was employed to determine the degrees of freedom (Satterthwaite 1946).

* $P < .05$.

*** $P < .001$.

**** $P < .0001$.

Pseudomonas syringae pv. *aesculi* can circulate in the xylem and phloem of infected horse chestnut trees and thus that bacteria may be moving freely within a tree (Green et al. 2009). Whether phage local adaptation at the level of the tree is driven by reduced gene flow or by divergent selection imposed by the trees (and the associated ecosystem) remains to be explored and will be an interesting avenue for future study. However, comparisons of phage infectivity from the inside and outside of leaves (see below) suggest that both processes are important.

Phages from both the surface and the interior of leaves were found to be locally adapted, but these microenvironments differed in regard to the degree of local adaptation observed (fig. 3*A*). Overall, we found that phages from the leaf interior were more clearly adapted to local bacterial populations than were those from the leaf surface, a result that could indicate either increased resistance of surface bacterial isolates or decreased phage prevalence on the leaf surface. This latter explanation is somewhat less supported in light of the surprisingly high prevalence of *P. syringae*–specific phages on the leaf surface (fig. 2*C*) despite the low frequency of pseudomonad isolates found there (fig. 2*A*). Perhaps a more likely explanation is that, unlike phages within the leaf interior, phages on the leaf surface are unable to successfully adapt to infect their local host populations; this result is in line with our predictions because phage populations infiltrating the leaf should experience a lower migration load, interact with a more constant bacterial host population, and have increased persistence by escaping the harmful effects of ultraviolet light exposure. For example, phages have been shown to be very sensitive to UV light and are likely to be short-lived in the environment relative to more sheltered within-host environments (Gill and Abedon 2003; Iriarte et al. 2007). Importantly, the observed patterns of local adaptation cannot be explained strictly by differences in species composition inside and outside the leaf and across trees (which were considerable in both cases). The genus-specific analyses suggest that the decreased local adaptation found for surface microenvironments is not simply a result of more-susceptible genera inhabiting the interior of the leaflet (although we cannot rule out the possibility that the microenvironments harbor different bacterial genotypes) but rather that interior microenvironments might be more conducive to phage adaptation (fig. 3).

Together these results are indicative of phage adaptation at both the community level (i.e., adapting to common bacterial species within the plant host) and the within-species level (i.e., differentially adapting to local parasite species/genotypes within a given genus/species). In addition, we found no evidence that bacterial populations were locally adapted to phages (i.e., that they were more resistant to local phages than foreign phages or more resistant to their own phages than other bacterial populations), sug-

Table 2: Variation in proportion of interior bacterial isolates (out of 20 tested per leaflet) that were susceptible to phage inocula from the leaf interior in relation to their source tree and source leaf (nested within tree)

Source	df	MS	F	Partial η^2
Bacteria tree	7	.48	.67	.40
Bacteria tree error	7	.07		
Phage tree	7	.11	1.15	.42
Phage tree error	8	.10		
Bacteria tree × phage tree	49	.04	2.81****	.13
Local adaptation across trees	1	.22	84.35****	.64
Bacteria tree × phage tree error	929	.01		
Bacteria leaf within tree	7	.07	5.46****	.04
Bacteria leaf within tree error	929	.01		
Phage leaf within tree	8	.10	1.64	.47
Phage leaf within tree error	15	.06		
Bacteria leaf × phage leaf within tree	15	.06	4.53****	.07
Local adaptation across leaves within tree	1	.09	1.82	.02
Bacteria leaf × phage leaf within tree error	929	.01		

**** $P < .0001$.

gesting that the bacteriophages within the leaf may be ahead in the coevolutionary battle, as would be predicted by their typically higher migration and mutation rates, larger population sizes (Lively 1999; Gandon 2002; reviewed in Greischar and Koskella 2007), and the asymmetrically higher selection pressure for infectivity, relative to that for host resistance (e.g., Morgan et al. 2005; Salathé et al. 2008). This might also be indicative of a trade-off between phage resistance and an ability to infect plant hosts for bacterial populations within the leaf; these bacterial populations may have a reduced ability to respond to selection from local phages because of alternate and relatively stronger selection imposed by the plant host immune response. Like those of other studies examining the spatial scale of local adaptation, our results underscore the key role that gene flow plays in homogenizing populations (Ebert 1994; Mopper et al. 1995; Thrall et al. 2002; Cogni and Futuyma 2009). However, unlike previous studies, our work shows that the local biotic environment plays a critical role in shaping the magnitude and spatial scale of parasite adaptation.

Our results extend the small but growing body of work documenting that parasite local adaptation in coevolving bacteria and phages occurs in natural populations (Held and Whitaker 2009; Vos et al. 2009; Rodriguez-Brito et al. 2010) and is likely a ubiquitous interaction driving evolutionary dynamics in bacterial populations (Buckling and Rainey 2002*b*; Morgan et al. 2005). It has long been known that phages have the potential to be strong selective agents in bacterial populations, because they lyse susceptible host cells and decrease population sizes (d'Herelle 1930; Lenski 1988). However, for this potential to be fulfilled, phages must also reach relatively high prevalence in bacterial populations and be able to adapt to specific, common bacterial species and genotypes. The results from this study demonstrate that lytic phages are both prevalent in the phyllosphere and able to adapt to both common bacterial species within the community and local populations of these species. Although we do not explicitly show coevolution in this study, in that we do not demonstrate a change in the bacterial populations in response to phages, the high infectivity of phages to bacteria suggests the potential for strong phage-imposed selection on bacteria.

In summary, our results emphasize that local adaptation of phages to plant-colonizing bacteria is highly sensitive to the plant host environment over very small spatial scales. The observed differences in phage adaptation across bacterial populations found only micrometers apart support the presence of small-scale selection mosaics in a tritrophic interaction, whereby one species (the host plant) is acting as the biotic environment influencing the interaction between the other two (phages and bacteria). Our data indicate that specific phage adaptation to local bacterial populations in the phyllosphere is sufficient to allow for strong reciprocal selection, a key assumption for the utility of phage therapy to regulate bacterial populations (Iriarte et al. 2007; Jones et al. 2007). The stronger signature of phage local adaptation inside the host leaf relative to the surface suggests that the strength of reciprocal selection between phages and bacteria and/or the degree of dispersal differs between these two microenvironments. This reinforces the developing idea that selection mosaics should be common across multitrophic interactions (Thompson 2005; Craig et al. 2007). More generally, these results highlight the importance of understanding the spatial scale and biotic complexity of species interactions in

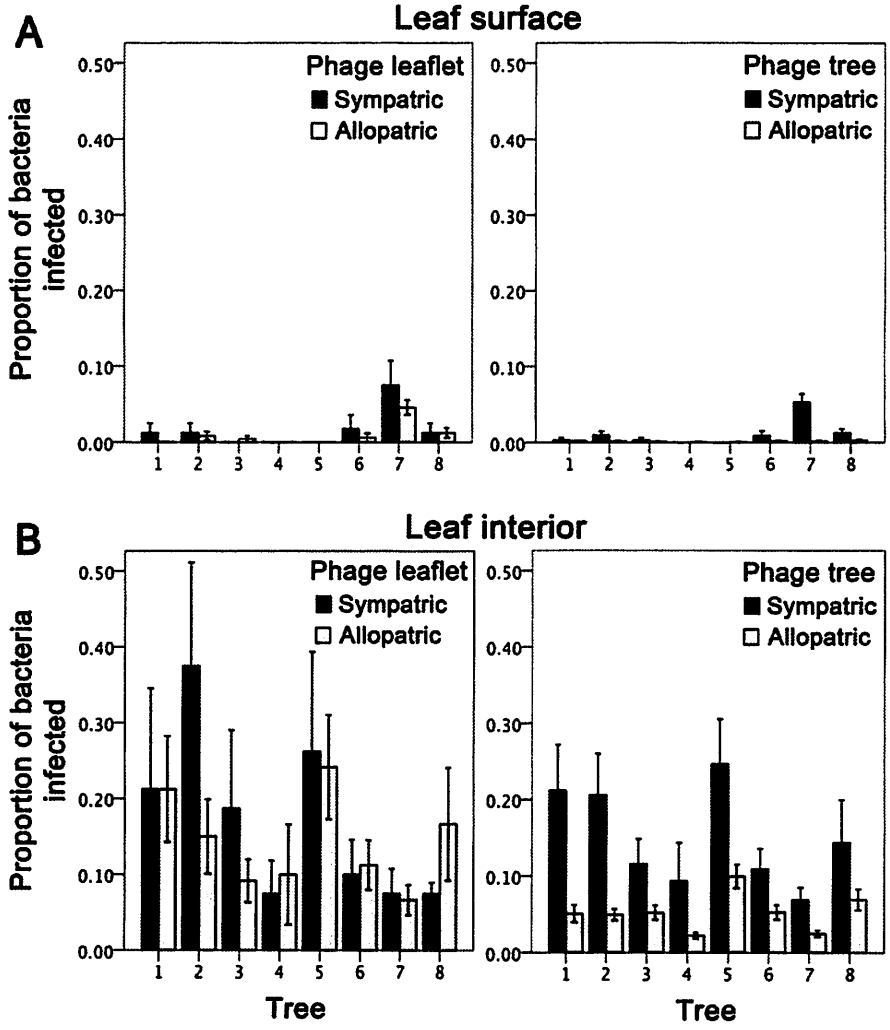

Figure 4: Phage local adaptation across microenvironments and spatial scales. *A*, Results for the surface cross-inoculation; *B*, results from interior cross-inoculation. Graphs on the left present the mean proportion of bacteria infected (out of 20 isolates per leaflet) for sympatric phage/bacteria combinations (from the same leaf) relative to allopatric combinations (from different leaves within the same tree). Graphs on the right present the mean proportion of bacteria infected (averaged over leaves within tree) for sympatric phage-bacteria combinations (from the same tree) relative to allopatric combinations (from other trees).

successfully predicting the outcome of coevolution (Taylor et al. 1998; White and Gilligan 1998; Morozov et al. 2007).

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Appendix from Koskella et al., "Local Biotic Environment Shapes the Spatial Scale of Bacteriophage Adaptation to Bacteria"

(Am. Nat., vol. 177, no. 4, p. 000)

Common-Garden Experiment Testing Natural Phage Populations against Previously Characterized Strains of *Pseudomonas syringae*

We performed a cross-inoculation experiment in which all phage inocula (64 in total) were tested across 24 previously characterized pathovars (table A1) in order to further characterize the phages present in the horse chestnut phyllosphere.

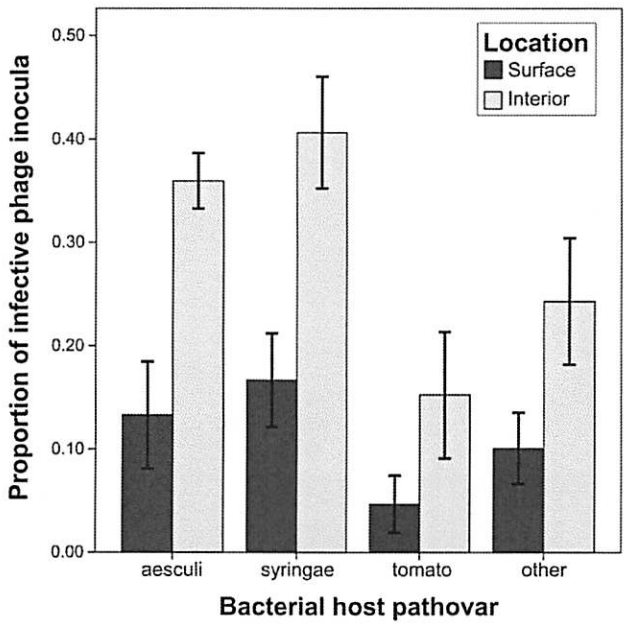

Figure A1: Proportion of the 32 phage inocula from the leaf surface (*dark gray*) and the 32 phage inocula from the leaf surface (*light gray*) that were able to infect each of the 24 previously characterized *Pseudomonas syringae* bacterial isolates, according to pathovar. The inocula from the leaf surface are less infective overall to *P. syringae* isolates (main effect of host source: $F = 18.347$, $P < .001$), and the pathovars differ in their susceptibility to phages from both the leaf surface and the interior (main effect of host pathovar: $F = 4.052$, $P = .013$; but no interaction effect between pathovar and phage source: $F = 0.619$, $P = .607$). Bars represent means ± 1 SEM.

Table A1. List of previously characterized *Pseudomonas syringae* pathovars used in the common-garden experiment

Pathovar, designation	Host	Source or reference
aesculi:		
6617	Horse chestnut	Forest Research (Northern Research Station, Roslin)[a]
6620	Horse chestnut	Forest Research (Alice Holt Lodge, Farnham, Surrey)
6623	Horse chestnut	Forest Research (Alice Holt Lodge, Farnham, Surrey)[a]
6631	Horse chestnut	Alain Bultreys, Belgium
aptata:		
NCPPB3539	Sugar beet	J. E. Sellwood
avellanae:		
592	Hazelnut	ISPaVe[b]
593	Hazelnut	ISPaVe[b]
glycinea:		
R4a	Soybean	Kobayashi et al. 1990
49a/90	Soybean	Ullrich et al. 1993
4180	Soybean	Bender et al. 1993
maculicola:		
M4	Radish	Debener et al. 1991
phaseolica:		
1448A	Kidney bean	Joardar et al. 2005
1449B	Hyacinth bean	Taylor et al. 1996
syringae:		
61	Bean	Huang et al. 1988
B728a	Snap bean	Feil et al. 2005
B301D	Pear	Cody and Gross 1987
tabaci:		
ATCC 11528	Tobacco	American Type Culture Collection
tomato:		
DC3000	Tomato	Buell et al. 2003
PT23	Tomato	Bender et al. 1986
08241	Tomato	Christine Smart
A9	Tomato	Christine Smart
519	Tomato	Omnilytics
BM 192	Tomato	Omnilytics
DC97T24A	Tomato	Omnilytics

[a]From Green et al. (2010).
[b]Culture Collection of Istituto Sperimentale per la Patologia Vegetale, Rome.

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