

ORIGINAL ARTICLE

Labile associations between fungus-growing ant cultivars and their garden pathogens

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The distribution of genetic and phenotypic variation in both hosts and parasites over their geographic ranges shapes coevolutionary dynamics. Specifically, concordant host and parasite distributions facilitate localized adaptation and further specialization of parasite genotypes on particular host genotypes. We here compare genetic population structure of the cultivated fungi of the fungus-growing ant *Apterostigma dentigerum* and of the cultivar-attacking fungus, *Escovopsis*, to determine whether these microbial associations have evolved or are likely to evolve genotype–genotype specialization. Analyses based on amplified fragment length polymorphism (AFLP) genotyping of host cultivars and pathogenic *Escovopsis* from 77 *A. dentigerum* colonies reveal that populations of hosts and pathogens are not similarly diverged and that host and pathogen genetic distances are uncorrelated, indicating that genetically similar parasites are not infecting genetically similar hosts. Microbial bioassays between pathogens and cultivars of different genotypes and from different populations show little pairwise specificity; most *Escovopsis* strains tested can successfully infect all cultivar strains with which they are paired. These molecular and experimental data suggest that *Escovopsis* genotypes are not tightly tracking cultivar genotypes within the *A. dentigerum* system. The diffuse nature of this host–pathogen association, in which pathogen genotypes are not interacting with a single host genotype but instead with many different hosts, will influence evolutionary and ecological disease dynamics of the fungus-growing ant–microbe symbiosis.

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Introduction

Parasites specialize on hosts at numerous ecological scales. At the broad level, most parasites attack only particular taxonomic host groups (for example, birds, annual plants, bacteria); at the finest level, some pathogen genotypes are specialized at attacking only a narrow range of host genotypes (Carius *et al.*, 2001; Schulenburg and Ewbank, 2004). Specialization is a consequence of reciprocal adaptation by sympatric hosts and parasites. As predicted by the Red Queen hypothesis, this can lead to local parasite adaptation, the increased ability of parasites to infect and utilize local hosts (Parker,

1985; Lively, 1989; Gandon *et al.*, 1996; Kaltz and Shykoff, 1998; Lively and Dybdahl, 2000; Dybdahl and Storfer, 2003).

Whether specialization arises is contingent on a variety of complex ecological and evolutionary factors. In particular, gene flow across host and parasite metapopulations is expected to play an important role in the evolution of localized adaptation within populations (Price, 1980; Kaltz and Shykoff, 1998; Lively, 1999; Thompson, 1999; Gandon and Michalakis, 2002; Thompson and Cunningham, 2002; Criscione *et al.*, 2005). In general, host and parasite migration among populations can provide novel genetic variation that natural selection can subsequently act on, creating the potential for local adaptation, but it can also counteract adaptation by introducing locally maladapted alleles (Slatkin, 1985). More specifically, theory predicts that it is the relative gene flow of hosts and parasites that is important in defining local adaptive processes. First, when both hosts and

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their parasite have low gene flow, and particularly when their patterns of gene flow are similar, local parasite adaptation is likely to arise (Price, 1980; Kirkpatrick and Barton, 1997; Gandon, 1998). When parasites migrate more freely than their hosts (that is, parasite populations are less genetically structured than host populations), parasites are also expected to be locally adapted to hosts (Kaltz and Shykoff, 1998), but when hosts experience higher gene flow than parasites, parasites may be better suited to attack allopatric hosts over sympatric ones, thus appearing locally maladapted (Gandon *et al.*, 1996).

Therefore, given that gene flow plays a critical role in coevolutionary dynamics, to understand the evolution of host–parasite interactions, it is important to have information on the relative population structure of the two players within a system. To date, only a handful of studies have characterized the population genetic structure of natural host and parasite metapopulations across their geographic range (Mulvey *et al.*, 1991; Dybdahl and Lively, 1996; Davies *et al.*, 1999; Delmotte *et al.*, 1999; Martinez *et al.*, 1999; Jobet *et al.*, 2000; Sire *et al.*, 2001; Jerome and Ford, 2002; Johannesen and Seitz, 2003), a very small fraction of which involve microbial pathogens (Delmotte *et al.*, 1999). Jobet *et al.* (2000), for example, found similar genetic differentiation between populations of the urban cockroach (*Blattella germanica*) and its nematode parasite (*Blatticola blattae*). Many other studies, however, have found asymmetrical patterns of genetic structuring in hosts and parasites. Delmotte *et al.* (1999), for instance, found that populations of the fungal pathogen *Microbotryum violaceum* were much more strongly differentiated than were populations of its host plant, *Silene latifolia*, while Dybdahl and Lively (1996) found that populations of trematode parasites (*Microphallus* sp.) were much less differentiated than those of their host snails (*Potamopyrgus antipodarum*).

The fungus-growing ant–microbe symbiosis provides a unique opportunity to study microbial host–pathogen population structure, parasite specificity and disease dynamics. In this insect agricultural system, more than 200 ant species in the Tribe Attini are known to cultivate fungus as their primary food source. When new colonies are formed, founding queens take a piece of fungus from their mother's colony to start new colonies. Each colony cultivates a single fungal strain (Poulsen and Boomsma, 2005; Gerardo, personal observation; Scott *et al.*, manuscript in preparation), either in the family Lepiota-ceae or Pterulaceae (Basidiomycota, Agaricales) (Chapela *et al.*, 1994; Munkacsi *et al.*, 2004). These cultivars are attacked by *Escovopsis*, a genus of microfungi (Ascomycota, Hypocreales) thus far only found in association with fungus-growing ant colonies (Currie *et al.*, 1999; Reynolds and Currie, 2004). Though only two species of *Escovopsis*, *E. Weberi* and *E. aspergilliodes*, are currently formally

described (Seifert *et al.*, 1995), morphological and genetic diversity of *Escovopsis* strains suggests that the genus is comprised of many species (Gerardo *et al.*, 2006a). Analyses at the broad, interspecific level indicate that the diverse *Escovopsis* types, as defined by morphology and genetics, are specialized: particular *Escovopsis* clades only attack specific clades of fungal cultivars (Currie *et al.*, 2003; Gerardo *et al.*, 2004, 2006a). This specificity is not maintained by transmission along with the host cultivar, as infection patterns suggest that *Escovopsis* spp. are horizontally transmitted via a yet unknown mechanism (Currie *et al.*, 1999; Currie, 2001; Gerardo *et al.*, 2004).

The outcome of cultivar–*Escovopsis* encounters, as well as other host–parasite interactions, is ultimately predicated upon adaptations on part of the host to resist parasite establishment and adaptations on part of the parasite to overcome host defenses and establish infection (Combes, 2004). In many systems, adaptive mechanisms on the part of the host and parasite are difficult to elucidate. In the fungus-growing ant–microbe symbiosis, however, recent studies of the microbes associated with the ant genus *Apterostigma* suggest mechanisms by which host cultivars can prevent some infections and mechanisms by which pathogenic *Escovopsis* can efficiently infect some hosts. Experimental bioassays interacting *Escovopsis* with cultivars raised by a range ant species indicate that these pathogens are attracted to chemical signals produced by their host cultivars and by closely related fungi (Gerardo *et al.*, 2006b). This attraction to cultivar signals may increase the efficiency by which *Escovopsis* establishes infection. Conversely, the same *Escovopsis* strains are not attracted to, but are inhibited by, distantly related cultivars raised by other fungus-growing ants. These phenotypes, *Escovopsis* host-seeking and cultivar resistance, may limit this parasite to successfully infecting only the cultivars raised by a subset of fungus-growing ant species (Gerardo *et al.*, 2006b). It is unknown whether these adaptive mechanisms could function to maintain tight intraspecific specificity of *Escovopsis* strains on strains of its natural host.

To date, we know little about intraspecific host–pathogen population dynamics in the fungus-growing ant symbiosis, as the few fine-scale genetic analyses of fungus-growing ant-associated microbes have focused only on the host cultivars in a single geographical region (in and around the Panama Canal zone) (Bot *et al.*, 2001; Green *et al.*, 2002; Poulsen *et al.*, 2005; Mikheyev *et al.*, 2007), rather than on both host and parasites across populations. Here, we use amplified fragment length polymorphism (AFLP) genotyping to characterize the population genetic structure of cultivars and *Escovopsis* pathogens isolated in tandem from the colonies of *Apterostigma dentigerum* over a large geographic-scale spanning Central and South America. We use these population genetic data to compare patterns of

host and parasite genetic structure. We also examine the distribution of host and parasite genotypes to look for signatures of fine-scale parasite specialization. These molecular analyses are coupled with experimental bioassays pairing genetically diverse parasite and cultivar strains in an attempt to identify signatures of local adaptation and strain–strain specificity. By assessing both patterns of host and parasite distribution over large geographic scales and specificity of host–parasites interactions at finer scales, we gain insight into the evolutionary and ecological dynamics of the *Apterostigma* ant–microbe symbiosis.

Materials and methods

Study system and collecting

Colonies of *A. dentigerum* are common along stream banks and under logs throughout much of Central and South America (Gerardo, personal observation). Unlike subterranean ant colonies, *A. dentigerum* colonies can be easily detected and identified because of a conspicuous white veil of fungus that protects their internal fungal garden (Figure 1a; Villesen *et al.*, 2004), facilitating collection of both garden material and ants from many colonies across populations.

All *A. dentigerum* ants raise cultivars in the family Pterulaceae (Basidiomycota, Agaricales), which, based on DNA sequence analysis, appear to be genetically similar cultivar strains (Villesen *et al.*, 2004). Within the context of a larger set of *Apterostigma* cultivar sequences, *A. dentigerum* cultivars are monophyletic at two genes (25S rDNA and COI) but not at ITS internal transcription spacer, a hypervariable marker. Although species boundaries have not been subscribed to fungal cultivars, the G2, G3 and G4 groups, in which *Apterostigma* cultivars are divided, are each considered well-supported monophyletic clades, and genetic differences within these clades, including the G2 group to which all *A. dentigerum* cultivars belong, are referred to as ‘strains’ or ‘types’ (Villesen *et al.*, 2004) rather than as ‘species’.

These *A. dentigerum* cultivar strains are attacked frequently by three genetically and morphologically distinct parasite types: brown-spored, yellow-spored and white-spored *Escovopsis* (Gerardo *et al.*, 2006a). All three *Escovopsis* types form monophyletic clades with little sequence divergence and are specific to attacking *Apterostigma* spp. cultivars (Gerardo *et al.*, 2006a). Based on the morphological and sequence similarity within each clade, we refer to each *Escovopsis* type as a ‘species’.

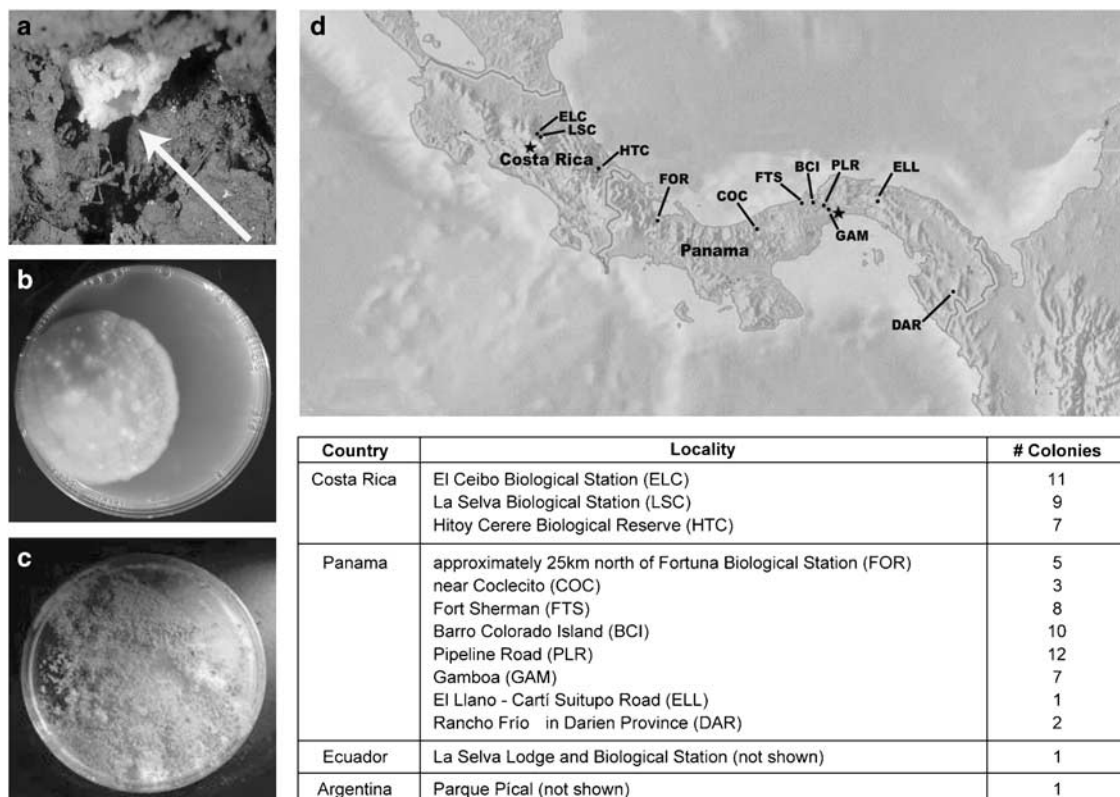


Figure 1 Sampling of *A. dentigerum* colonies. (a) *A. dentigerum* colonies are easily located in the field because of the conspicuous, white fungal veil (see arrow) that protects their garden. After collection for this study, gardens were sampled to obtain pure isolates of the ants cultivated fungi (b) and the parasitic fungus *Escovopsis* (c). (d) Collecting sites and sample sizes. Cultivar and *Escovopsis* were collected from 77 colonies throughout Costa Rica, Panama, Ecuador and Argentina (the latter two are not shown). Stars mark the country capitals.

Garden material from 472 *A. dentigerum* colonies was collected in 2001–2003 in Central and South America at 13 localities (Figure 1). At least 10 garden pieces ($\sim 8\text{ mm}^3$) from each colony were grown on potato dextrose agar (PDA; Difco, Detroit, MI, USA) with bacteria-inhibiting antibiotics (50 mg/l each of penicillin and streptomycin), and pieces identified as either cultivar or as *Escovopsis* were subsequently subcultured to obtain axenic (pure) cultures before storage at -80°C .

From 77 of the 472 colonies collected, axenic cultures of both cultivar (Figure 1b) and brown-morphotype *Escovopsis* (Figure 1c) were isolated and stored successfully. Only samples from these colonies were included here, because use of host–parasite pairs isolated in tandem (that is, cultivar and *Escovopsis* from the same colony) assured that both hosts and pathogens were sampled similarly across space and time. Sample sizes for each population are listed in Figure 1. *Escovopsis* isolates of the yellow-spored and white-spored morphotypes were not included in the study, because $\text{Ef-1}\alpha$ sequences indicate that they are genetically distinct species from the brown-morphotype *Escovopsis*, and, furthermore, these pathogens are less common than the brown-morphotype, limiting sample size (Gerardo *et al.*, 2006a).

AFLP genotyping

To examine the distribution of host and pathogen genotypes both across populations and relative to one another, we used AFLPs to profile cultivars and *Escovopsis* isolated from the same 77 colonies. Preceding amplification, DNA from a single cultivar and a single *Escovopsis* isolate from each colony was extracted according a cetyl trimethyl ammonium bromide protocol modified from Bender *et al.* (1983). Cultivar and parasite isolates from two randomly selected colonies were extracted twice and genotyped separately to detect the amount of noise (unreliable marker differences) generated during the amplification and scoring process. These duplicates were not included in graphical or statistical analyses.

For all samples, reactions followed the AFLP protocol for small plant genomes (www.appliedbiosystems.com; protocol 4303146) with the following modifications found to increase reliability of AFLP reactions: (1) before preselective amplification, $120\ \mu\text{l}$ of $\text{TE}_{0.1}$ was added to the restriction-ligation product instead of the recommended $189\ \mu\text{l}$, (2) the preselective product was not diluted for selective amplification, (3) for both preselective and selective amplifications, 25 thermocycler cycles were used instead of the recommended 20 cycles, (4) $3\ \mu\text{l}$ of each selective product was mixed with $0.4\ \mu\text{l}$ of GeneScan 500 Rox and $6.6\ \mu\text{l}$ of Hi-Di formamide before generation of AFLP markers on an ABI Prism 3100 Genetic Analyzer. For selective amplifications, six combinations of AFLP-primer extensions were

chosen because they generated high levels of polymorphic markers that could be scored reliably: AC/CAA, AC/CTG, AC/CTC, TG/CAG, TG/CTC and TC/CAG. Markers were scored blindly in Genotyper 2.5 by simultaneously comparing all fragments of a given length across all 77 *Escovopsis* isolates and, in a separate analysis, across all 77 cultivar isolates. Only markers that could be scored as unambiguously present/absent across all pathogen or host samples were used in analyses. At all stages, all cultivar and *Escovopsis* samples were run at the same time in 96-well plates and using the same reagents to minimize experimental artifacts.

Host and parasite population structures

For analysis of population structure, we performed parallel analyses of the cultivar (host) and *Escovopsis* (parasite) datasets and then compared results between the two. We conducted two analyses of molecular variance (AMOVA) in Arlequin (Schneider *et al.*, 2000) to partition genetic variation both among host and among parasite isolates within and between localities. The AMOVA module in Arlequin generates Φ statistics, which is a molecular analog to Fisher's F_{st} (Excoffier, 2001). Population pairwise Φ_{st} values were also generated to determine the proportion of differences between hosts, and separately between parasites, associated with each locality. Levels of significance were determined through 100 000 random permutation replicates. Additional analogs of overall F_{st} (Θ^{B} and G_{st}^{B} , an estimate of Nei's G_{st} (Nei, 1977) for diallelic loci) were calculated using Bayesian methods implemented in the program Hickory v1.0.4 (Holsinger *et al.*, 2002). We ran all possible models available in Hickory, with default parameters recommended by the authors (Holsinger *et al.*, 2002), but there were no substantial differences, so we report values obtained using the full model. For all population analyses, we excluded three localities at which only a single sample was collected (LSE, ARG and ELL), because no within-locality variation could be determined.

For hosts, and separately for parasites, we conducted Mantel tests in ZT (Bonnet and Van de Peer, 2002) to determine correspondence between each pairwise Φ_{st} (genetic distance) matrix and a pairwise geographical distance matrix. A significant, positive correlation would indicate effects of isolation by distance. All Mantel tests mentioned hereafter were also conducted using ZT and were performed with 10 000 permutations. Pairwise linear geographical distances between localities were calculated using the program Range (Luetgert). We also plotted the relationship between pairwise Φ_{st} and geographical distance for all pairs of host populations and all pairs of parasite populations.

To determine whether host and parasite populations exhibited a similar spatial pattern of divergence, we used Arlequin to construct two matrices:

(1) the Nei's corrected average pairwise cultivar population differences; and (2) the Nei's corrected average pairwise *Escovopsis* population differences (Nei and Li, 1979). We then used a Mantel test to assess correspondence between the matrix of cultivar pairwise Φ_{st} values and parasite pairwise Φ_{st} values. A significant correlation would indicate that parasite populations show similar relative divergence to the host populations that they are attacking. We also visually compared cultivar population structure against parasite population structure using Structure v2.1 (Pritchard *et al.*, 2000). Structure uses multilocus genotypic data to evaluate models of different numbers of clusters (K), with each individual probabilistically assigned to a reconstructed genetic cluster based on its multilocus genotype and the allele frequencies estimated for each cluster. Each genotype was treated as effectively haploid, where each locus contained one of two alleles determined by the presence or absence of a band. We applied a model of no-admixture and allele frequencies were assumed to be correlated within populations. Simulations were run from $K=1$ to $K=9$ with each K iterated five times, and we did not include any priors regarding sample location. To select the appropriate K , we calculated a ΔK statistic according to Evanno *et al.* (2005). Structure was also used to calculate F_K values, interpreted as F_{st} analogs between each population and a single hypothetical common ancestral population (Pritchard *et al.*, 2000; Falush *et al.*, 2003). F_K values were obtained by running the no-admixture model with $K=10$, utilizing locality as the population origin. All Structure simulations began with a burn-in of 100 000 and a runtime of 300 000.

Association of host and parasite genotypes

Correlation between host and parasite population pairwise differences would indicate that more genetically similar populations of hosts and parasites are associated, but this would not reveal parasite specialization at finer levels (that is, whether each parasite genotype within a population is attacking a narrow range of host genotypes within a population). To determine whether genetically similar parasites are attacking genetically similar hosts, we first visualized the relationships between the 77 cultivar isolates using mean character distances (that is, the sum of marker differences between two samples/total number of markers), generated in PAUP* (Swofford, 2002), to construct a nonmetric multidimensional scaling (NMDS) plot using NCSS (ver. 2000, Hintze, 2001). NMDS is an ordination technique that detects nonhierarchical structure by reducing the multidimensional relationship between entities to a smaller number of dimensions. A similar plot was created for the pathogen isolates. Mean character distances were used for these and all subsequent analyses, because though Nei–Li restriction distances (Nei and Li,

1979) are often selected for AFLP data analysis, many *Escovopsis* pair distances were undefined using this method. The Nei–Li and mean character distances for the cultivars were highly correlated (Mantel test, $r=0.78$, $P<0.0001$), however, and the results of no analysis were changed if the cultivar Nei–Li distances were used in place of the mean character differences. To visually verify the clustering produced through NMDS, we also used PAUP* to construct unweighted paired group means analysis (UPGMA) dendrograms.

In addition to visual inspection, we conducted two separate statistical analyses. First, we used a Mantel test to assess correlation between the host and parasite mean character difference matrices. Significance would indicate that more genetically similar parasite isolates (those with smaller mean character differences) attack more genetically similar host isolates, both between and within populations. Second, in both the NMDS plot and the UPGMA dendrogram, cultivar isolates fell into the same six visually distinct clusters. To verify the genetic distinctiveness of these clusters, we assigned each cultivar isolate to a cluster (clusters 1–6) and then used AMOVA to determine the proportion of the genetic variation among cultivar isolates. Pairwise comparisons among clusters were also conducted. Then, to determine whether the host cluster with which an *Escovopsis* isolate is associated could explain genetic variation among parasite isolates, we assigned each *Escovopsis* isolate to the cluster in which its host belonged and used these groups as a basis for AMOVA. A significant overall Φ_{st} would indicate the proportion of *Escovopsis* variation attributable to their association with genotypically distinct host clusters. We also conducted pairwise comparisons to determine which parasite groups, as defined based on host genotype cluster, were genetically differentiable. Significant pairwise differences would indicate cases in which genotypically differentiable parasite groups are attacking genotypically differentiable hosts.

Experimental test for specificity

We conducted microbial bioassays, following procedures in Gerardo *et al.* (2006b), to see if there is variation in the outcome of interactions between a set of genetically and geographically diverse *A. dentigerum*-associated *Escovopsis* strains and a set of equally diverse *A. dentigerum*-associated cultivar strains. For each bioassay, we placed a single isolate of cultivar near the edge of a 9 cm Petri dish with PDA + antibiotics. After 1 week, we inoculated the center of each plate with a single *Escovopsis* isolate. Each of nine cultivar isolates was interacted with each of the same nine *Escovopsis* isolates for a total of 81 bioassays. Experimental strains were chosen to maximize geographic and genetic diversity. Bioassays were monitored for up to 2 months. Interactions were scored for presence/absence of inhibition by

the cultivar on *Escovopsis* growth and presence/absence of attraction by *Escovopsis* to the cultivar (Figure 5).

To determine whether the variation that we observed in interactions between *Escovopsis* and cultivar isolates was associated with either genetic differences or geographic distances, we conducted a series of Mantel tests looking for the correlation between various distance matrices. Correlations with geographic distances would be consistent with local adaptation, while correlations with genetic distance would indicate specialization on particular genotypes. Four interaction distance matrices were constructed. The first matrix consisted of the inhibition distances between each pair of the nine cultivar strains, where each inhibition distance ranged from 0 to 1 and increased 0.11 for each case in which the two cultivar strains had a different inhibition result with the same *Escovopsis* strain (that is, one cultivar inhibited the *Escovopsis* strain while the other did not). A second matrix consisted of *Escovopsis* inhibition distances; each inhibition distance ranged from 0 to 1 and increased 0.11 for each case in which the two *Escovopsis* strains had a different inhibition result with the same cultivar strain (that is, one *Escovopsis* strain was inhibited while the other was not). The third and fourth matrices were comprised of cultivar and *Escovopsis* attraction distances, which were determined similarly to the inhibition distances.

With the AFLP data, we constructed two genetic distance matrices using PAUP*: a mean character difference distance matrix for the nine experimental cultivar strains and a mean character difference distance matrix for the nine experimental *Escovopsis* strains. We also constructed two geographic distance matrices: a matrix of distances between collection sites of the cultivar strains and a matrix of distances between collection sites of the *Escovopsis* strains. We then used ZT to conduct Mantel tests to examine the correlation between matrices of (1) cultivar inhibition distances and cultivar genetic distances, (2) cultivar inhibition distances and cultivar geographic distances, (3) cultivar attraction distances and cultivar genetic distances, (4) cultivar attraction distances and cultivar geographic distances, (5) *Escovopsis* inhibition distances and *Escovopsis* genetic distances, (6) *Escovopsis* inhibition distances and *Escovopsis* geographic distances, (7) *Escovopsis* attraction distances and *Escovopsis* genetic distances and (8) *Escovopsis* attraction distances and *Escovopsis* geographic distances. To account for multiple tests, we applied a Bonferroni correction on all comparisons.

Results

AFLP diversity

For the host cultivars, a total of 804 AFLP markers were identified using the six primer systems; all are

polymorphic and 208 (26%) are autapomorphic. For pathogenic *Escovopsis*, a total of 933 AFLP markers were identified; all are polymorphic and 334 (36%) are autapomorphic. Both cultivars and *Escovopsis* samples are diverse; mean character differences between cultivar isolates range from 0.02 to 0.29 (mean = 0.16, s.d. = 0.05), and mean character differences between *Escovopsis* isolates range from 0.04 to 0.22 (mean = 0.14, s.d. = 0.04). Of the four duplicated samples, the mean character difference between duplicates is low, ranging from 0.02 to 0.07 (mean = 0.04, s.d. = 0.02), and the difference between cultivar duplicates and between parasite duplicates is similar.

Host and parasite population structures

While both cultivar and *Escovopsis* populations exhibit little genetic structure along geographically determined boundaries, cultivar populations are relatively more geographically structured than those of their parasite. Among cultivar isolates, 11% of the variation is attributable to between-population differences (AMOVA overall Φ_{st} = 0.11, Table 1a, Θ^B = 0.11, 95% CI 0.10–0.12), while 6%–7% of the variation among *Escovopsis* isolates is attributable to population differences (AMOVA overall Φ_{st} = 0.07, Table 1a, Θ^B = 0.06, 95% CI 0.10–0.12). Estimates of G_{st}^B are very similar for cultivar (G_{st}^B = 0.10, 95% CI 0.09–0.11) and *Escovopsis* (G_{st}^B = 0.05, 95% CI 0.04–0.06).

Cultivar and *Escovopsis* population structures are shaped by geography but are not significantly correlated to one another. A Mantel test of the correspondence between cultivar pairwise Φ_{st} values and pairwise geographic distances confirms the effect of geographic isolation by distance (r = 0.34, P = 0.04). *Escovopsis* exhibits similar correlation between population genetic and geographic distances (r = 0.38, P = 0.03). This positive relationship between population genetic and geographic distances is represented in Figure 2. There is not, however, significant correspondence between cultivar population pairwise Φ_{st} values and *Escovopsis* population pairwise Φ_{st} values (r = 0.22, P = 0.139). This absence of corresponding population structure between host and parasite can be visualized by comparing the genetic clusters of each symbiont (Figures 3a and 3b). Consistent with the pattern of *Escovopsis* being slightly less structured than its host, as determined by the F_{st} analogs above, the genetic structure of *Escovopsis* conforms best to a model of K = 3 genetic clusters, while the cultivar host is best explained by K = 6. Though discrepancy in the values of K between the cultivar and *Escovopsis* does not necessarily translate to incongruent population genetic structure, as it is possible for finer-scale cultivar genetic structure to be nested within the three clusters of *Escovopsis*, we did not observe such a clustering pattern. For the cultivar, F_K values (Figure 3c) are greatest in the Costa Rican

Table 1 AMOVA Φ_{st} values and pairwise comparisons for host and parasite localities

AMOVA results (population differences)	Cultivar (host) (a)			Escovopsis (parasite) (b)		
	Variance	d.f.	Total (%)	Variance	d.f.	Total (%)
Between-populations	7.3	9	11.1	4.8	9	7.4
Within-populations	58.9	64	88.9	60.5	64	92.6
	Overall $\Phi_{st} = 0.11, P < 0.01$			Overall $\Phi_{st} = 0.07, P < 0.01$		

Between-population pairwise Φ_{st} values Cultivar (below diagonal) and Escovopsis (above diagonal)										
	ELC	LSC	HTC	FOR	COC	FTS	BCI	PLR	GAM	DAR
ELC		0.01	0.06	0.04	0.05	0.06	0.28	0.13	0.06	0.06
LSC	0.13		0.03	0	0	0.01	0.2	0.05	0.01	0.01
HTC	0.32	0.02		0.01	0.04	0.05	0.31	0.11	0.06	0.23
FOR	0.26	0.002	0		0	0	0.24	0	0	0.30
COC	0.11	0.09	0.24	0.16		0.06	0.12	0.04	0.14	0.01
FTS	0.06	0	0.12	0.10	0		0.02	0	0	0.02
BCI	0.10	0.17	0.27	0.15	0.07	0.13		0.03	0.11	0.30
PLR	0.09	0.08	0.15	0.14	0	0	0		0.01	0
GAM	0.12	0.21	0.32	0.29	0	0.06	0	0		0.01
DAR	0.02	0.17	0.36	0.15	0.03	0.10	0.02	0.15	0.08	

Abbreviations: AMOVA, analyses of molecular variance; BCI, Barro Colorado Island; COC, Coclecito; DAR, Darien Province; d.f., degrees of freedom; ELC, El Ceibo Biological Station; FOR, Fortuna Biological Station; FTS, Fort Sherman; GAM, Gamboa; HTC, Hitoy Cerere Biological Station; LSC, La Selva Biological Station; PLR, Pipeline Road. Overall, Φ_{st} values indicate the proportion of variation attributable to host (a) and parasite (b) genotype differences between populations. Pairwise comparisons are between populations, with pairwise Φ_{st} values for cultivar below and for parasite above the diagonal. All P -values were derived by permuting genotypes among samples (100 000 permutations). Significant pairwise Φ_{st} values ($P < 0.05$) are in bold.

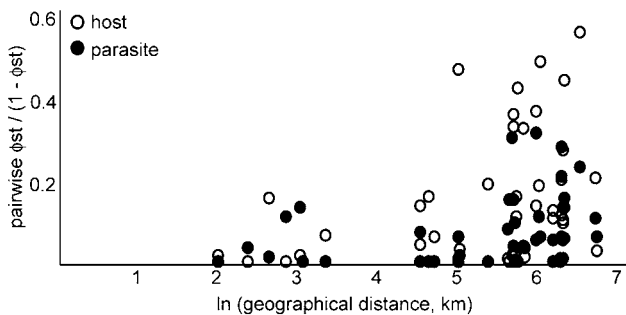


Figure 2 Isolation by distance. Plot of pairwise $\Phi_{st}/(1-\Phi_{st})$ against pairwise geographical distance between each of 10 populations of hosts (open circles) and parasites (filled circles). The relationship between genetic and spatial distances was assessed using Mantel tests and is significant for both cultivars ($r = 0.34, P = 0.04$) and *Escovopsis* ($r = 0.38, P = 0.03$).

population Hitoy Cerere Biological Station (HTC) ($F_K = 0.12$), followed by Fort Sherman in Panama ($F_{st}, F_K = 0.10$) while the most divergent *Escovopsis* population occurs in Barro Colorado Island (BCI, $F_K = 0.10$), the only region with a substantial F_K for the pathogen. This implies that the most divergent host populations sampled do not correspond to the most divergent parasite population sampled, but it is important to note that these values, in general, are relatively low.

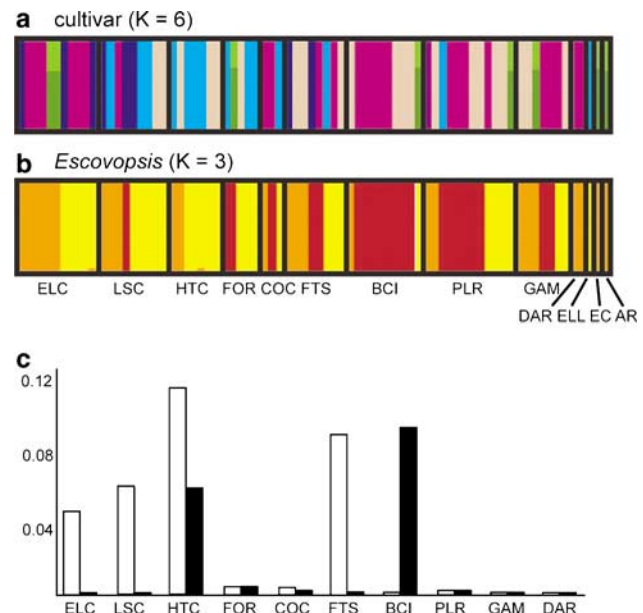


Figure 3 Host and parasite population differentiation. (a) Genetic clusters for cultivars ($K = 6$) and (b) for *Escovopsis* ($K = 3$), determined using Bayesian methods in Structure and a ΔK statistic of Evanno *et al.* (2005). Each vertical line represents a single individual and each color corresponds to the genetic cluster to which an individual is probabilistically assigned. (c) F_K values (an F_{st} analog between each population and a hypothetical common ancestor of all populations) for cultivars (white) and *Escovopsis* (black). Populations are arranged in order from northern Costa Rica to eastern Panama.

Association of host and parasite genotypes

Cultivars exhibit substantial genetic structure, as seen through the clustering of isolates in both the NMDS plot (Figure 4a) and UPGMA dendrogram (not shown) of all cultivar samples. Both clustering algorithms group cultivar isolates into six main clusters, and all isolates fall into the same cluster in both analyses. For the NMDS plot, dimension one captures 29% of the cultivar variation and dimension two captures an additional 22%. Upon *a posteriori* assignment of each of the cultivars to one of the six genotypic clusters, the resulting clusters account for 54% of the variation among

isolates (Table 2a, AMOVA, overall $\Phi_{st} = 0.54$), substantially more than when the cultivars are assigned to populations (Table 1a, AMOVA, overall $\Phi_{st} = 0.11$). All six genotype clusters are significantly genetically distinct from one another (Table 2, pairwise differences). As can be seen in the Structure analysis (Figure 3b), these clusters do not correspond to geographically isolated populations.

Escovopsis exhibits less genetic structure, with little clustering in the NMDS plot (Figure 4b) or UPGMA dendrogram (not shown). For the NMDS plot, dimension one captures 37% of the isolate

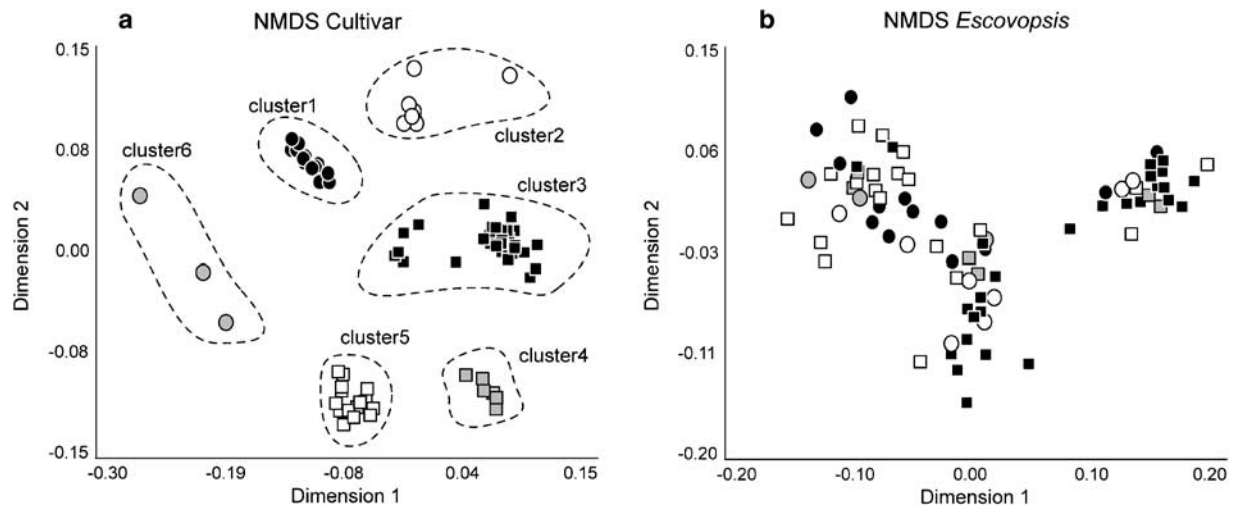


Figure 4 Clustering of all host and parasite isolates. (a) Nonmetric multidimensional scaling (NMDS) solution for all cultivar isolates. Dashed lines demarcate six main host genotype clusters. Pairwise Φ_{st} comparisons indicate that all clusters are genetically differentiable (Table 2). (b) NMDS solution for all *Escovopsis* isolates. Isolates are coded by the cluster (1–6) of their associated host.

Table 2 AMOVA Φ_{st} values and pairwise comparisons for host genotype clusters and their associated parasites

AMOVA results (host clusters)	Cultivar (host) (a)			Escovopsis (parasite) (b)		
	Variance	d.f.	Total (%)	Variance	d.f.	Total (%)
Between-host clusters	40.4	5	54.1	1.64	5	2.53
Within-host clusters	34.3	71	45.9	63.13	71	97.47
	Overall $\Phi_{st} = 0.54$, $P < 0.001$			Overall $\Phi_{st} = 0.025$, $P = 0.048$		

Between-host cluster pairwise Φ_{st} values

Cultivar (below diagonal) and Escovopsis (above diagonal)

	Cluster1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Cluster1		0.04	0.08	0	0	0
Cluster2	0.66		0.02	0	0.01	0.06
Cluster3	0.54	0.46		0	0.03	0.08
Cluster4	0.77	0.71	0.42		0	0
Cluster5	0.61	0.59	0.50	0.52		0
Cluster6	0.60	0.49	0.43	0.61	0.49	

Abbreviations: AMOVA, analyses of molecular variance; d.f., degrees of freedom.

Overall Φ_{st} values indicate the proportion of cultivar genotypic variation that is captured by *a posteriori* assignment of each host to a genotype cluster (a) and the proportion of *Escovopsis* genotypic variation that is captured by assignment of parasites to their respective host clusters (b). Pairwise comparisons below the diagonal are between each cultivar cluster, and pairwise comparisons above the diagonal are between groups of *Escovopsis* isolates assigned to their hosts' clusters. All P -values were calculated by permuting genotypes among samples (100 000 permutations). Significant pairwise Φ_{st} values ($P < 0.05$) are in bold.

variation, and dimension two captures an additional 15%. Lesser parasite clustering in Figure 4 as compared to host clustering suggests that there may be little concordance between host genetic similarity and parasite genetic similarity, and absence of tight parasite tracking of hosts genotypes is confirmed by a lack of significant correspondence between mean host differences and their respective mean parasite differences ($r=0.04$, $P=0.065$).

When *Escovopsis* isolates were assigned to groups based on the genotypic cluster with which their host cultivar was associated, this clustering explains a small, though significant portion of the variation among parasite isolates (Table 2b, overall $\Phi_{st}=0.025$). Pairwise comparisons between groups of *Escovopsis* isolates that attack the different host genotype clusters indicate only one significant difference: parasites attacking cultivars in cluster one are only slightly, though significantly, genotypically distinct from parasites attacking cultivars in cluster three (Table 2, pairwise differences).

Experimental test for specificity

In the vast majority of bioassays, the *Escovopsis* isolate was attracted to the cultivar isolate, and the cultivar isolate was not able to inhibit the *Escovopsis* isolate (Figure 5). Of the eight Mantel tests looking for a correlation between an interaction distance matrix and either a genetic or geographic distance matrix, only one test had a P -value less

than 0.1; there is a negative correlation between cultivar attraction distance and cultivar geographic distance (simple Mantel test: $r=-0.3$, $P=0.04$), likely driven by the fact that one cultivar isolate from Pipeline Road (PLR) inhibited an *Escovopsis* isolate not inhibited by the other two PLR cultivar strains. This weak correlation is not significant when a Bonferroni correction is applied.

Discussion

The distribution of parasite genotypes relative to their hosts, when analyzed over large geographic scales, gives insight into both local and global disease dynamics. Here, at a broad geographic scale, we see that host and parasite populations are not similarly differentiated. First, there is no correspondence between host and pathogen population pairwise Φ_{st} values, suggesting that hosts and pathogen populations are not diverging in tandem. Second, the most divergent host populations (indicated by F_K) are not the same as the most divergent pathogen populations (Figure 3c). Furthermore, at a finer scale, we see no evidence of specialized pairings of host and parasite genotypes. Genetic analyses of neutral markers indicate that there is no correspondence between a matrix of host mean character differences and a matrix of pathogen mean character differences, and, whereas cluster analyses suggest several genetically distinct host clusters, there is no corresponding divergence in pathogen isolates (Figures 3 and 4). Supporting this lack of genotype–genotype specificity, experimental bioassays indicate that genetically distinct *Escovopsis* isolates are able to successfully infect a range of genetically and geographically diverse cultivar isolates (Figure 5), and there is no correspondence between genetic similarity of hosts and/or of parasites and infection success.

Many life-history traits of hosts and pathogens critically shape disease processes and likely lead to the patterns we see in the cultivar–*Escovopsis* system. Specifically, different modes of transmission increase the likelihood that parasite and host genotypes do not stay coupled across space and time (Huyse *et al.*, 2005). In the fungus-growing ant system, previous epidemiological studies have suggested that while cultivars are vertically transmitted by the ants from a mother’s colony to new colonies *Escovopsis* is independently horizontally transmitted (Currie *et al.*, 1999). It has been hypothesized that *Escovopsis* could be vectored between colonies by microarthropods, or could be acquired by foraging ants either from the environment or during encounters with other ants (Currie, 2001; Gerardo *et al.*, 2004). These mechanisms of disease spread likely have different barriers to gene flow than vertical transmission of the cultivar by the ants, and our results corroborate the existence of different barriers to gene flow for the host and parasite.

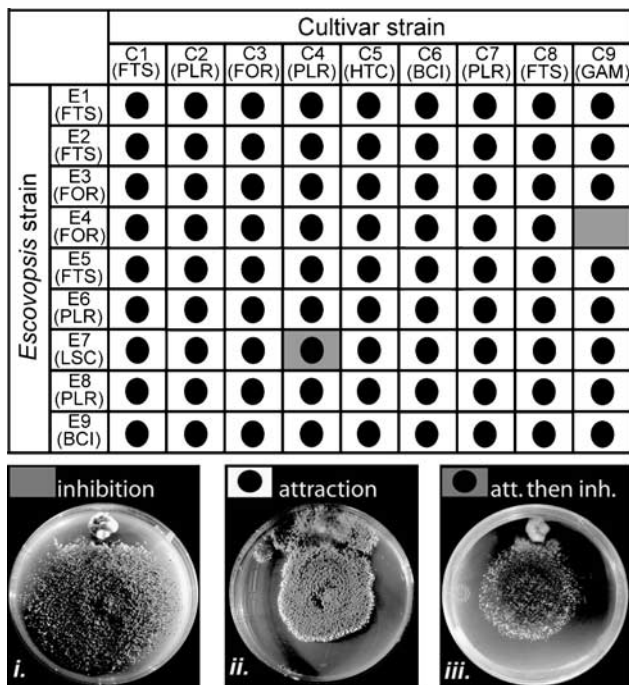


Figure 5 Microbial bioassays. Each cell represents the outcome of the interaction between one cultivar and one *Escovopsis* isolate. Gray indicates inhibition (i), a dot indicates attraction and subsequent infection (ii) and gray with a dot indicates attraction followed by inhibition (iii). The collection locality of each fungal strain is indicated in parentheses under its sample name.

Specifically, based on F_K values (Figure 3c), the most divergent host population (HTC) in Costa Rica's Talamanca mountains, is not the most divergent parasite population (BCI), an island in the Panama Canal (Figure 1). While water would provide a barrier to *Escovopsis* migration to BCI by any of the above-mentioned horizontal transmission mechanisms, it would not prevent dispersal of the host cultivar by winged, migrating ants, which could easily fly the short distance from BCI to the mainland. On the other hand, cultivar-dispersing ants may have difficulty migrating to and from HTC because of their sensitivity to cooler temperatures in such higher-elevation regions. If different barriers to gene flow exist, then parasite genotypes would be less able to tightly track host genotypes.

Even without coupled dispersal, tight congruence of host–parasite population structures can be maintained by parasite specificity, such that parasites are only able to establish in a population if the appropriate host genotype is present. For example, the global population structure of *Mycobacterium tuberculosis*, which causes tuberculosis in humans, is defined by six lineages each associated with different human populations. In urban areas where different human populations have recently become sympatric, the *M. tuberculosis* strains historically associated with these human populations are present. This matching of human and pathogen distributions is shaped in part by tandem host–pathogen dispersal but also by coevolutionary adaptation. In a diverse urban host population, *M. tuberculosis* strains are much more likely to be infective in humans from populations with which they coevolved than in humans from other lineages (Gagneux *et al.*, 2006). It is likely that specific host and parasite traits, namely host immune-related phenotypes and parasite virulence genes, maintain this specificity. We hypothesized that specific cultivar and *Escovopsis* traits would also maintain tight specificity of *Escovopsis* strains on cultivar strains. Host cultivars are known to be able to produce antibiotics that inhibit the growth of some *Escovopsis* spp., and *Escovopsis* spp. are known to efficiently recognize and establish infection on some hosts species (Gerardo *et al.*, 2006b). Results here, however, suggest that these mechanisms are not facilitating genotype–genotype specificity or local adaptation at a population level, as a range of genetically and geographically diverse *Escovopsis* strains were able to successfully infect a range of genetically and geographically diverse cultivar strains. Thus, these previously identified adaptive mechanisms (Gerardo *et al.*, 2006b) may prevent a given *Escovopsis* from switching between host species but do not necessarily prevent *Escovopsis* from switching between different host strains.

The patterns of host and parasite association that we see here, driven by relatively independent host–parasite dispersal and a lack of genotype–genotype specificity, have implications for the coevolutionary

dynamics of the cultivar–*Escovopsis* association. Specifically, focusing on the likelihood of *Escovopsis* to become adapted to local cultivar strains, theory suggests higher gene flow between parasite populations than between cultivar populations, particularly when coupled with substantial genetic structuring across host populations, will facilitate local adaptation of parasites on hosts (Gandon *et al.*, 1996; Gandon and Michalakis, 2002). Although *Escovopsis* does exhibit slightly higher gene flow than cultivars, this discrepancy is likely not sufficient to support local adaptation, as the host cultivars exhibit little genetic population structure. As a comparison, in systems where population genetic analyses detected more extensive parasite than host migration and experimental work confirmed that parasites perform better on local than on novel host genotypes, the relative difference between parasite and host migration rates was much greater than is seen in this study. For example, Dybdahl and Lively (1996) found much higher levels of gene flow in trematode parasites relative to their snail hosts, and it has been experimentally demonstrated that these parasites are locally adapted to common host genotypes (Lively, 1989; Lively and Dybdahl, 2000). Similarly, Mutikainen and Koskela (2002) found higher parasite gene flow in parasitic plants than their perennial hosts, and these parasites had been previously reported to be locally adapted to their hosts (Koskela *et al.*, 2000). In these cases, host populations were respectively 10 and 3 times more differentiated than their parasites, whereas here, with cultivar and *Escovopsis*, overall Φ_{st} values of host and parasite populations are less than twofold different. Moreover, results of Mantel tests between genetic and geographic distances indicate that the effects of isolation by distance in cultivars and *Escovopsis* are similar (Figure 3), and suggest that though there may be less parasite population differentiation at distances greater than 150 km (that is, when the natural log of geographic distance is greater than 5, Figure 3), at smaller distances, there is equal mixing between host populations as between parasite populations. Therefore, extensive local adaptation may be less likely in the cultivar–*Escovopsis* system than in other host–parasite associations where the difference between parasite and host genetic structure is greater.

Overall, it seems that local adaptation and strain-specific specialization is not likely to arise in the *A. dentigerum* cultivar–*Escovopsis* system. This is in contrast to the specificity that we see at broader scales, where *Escovopsis* species are constrained to attacking particular host species or groups of closely related species. This pattern of fine-scale lability underlying broad-scale specificity is consistent with other coevolutionary interactions. For example, a recent study of the host range of tropical plant pathogens found fungal pathogens were likely to be able to infect a range of closely related hosts, but

were less likely to infect plants distantly related to their host. In other words, the likelihood of infection establishment decreased with increasing phylogenetic distance between the source host plant and the target plant species, with the steepest decline occurring in the most closely related plant pairs (Gilbert and Webb, 2007). Another example is the mutualistic association of sepiolid squid and their bioluminescent bacteria of the genus *Vibrio*. In this mutualism, phylogenetic congruence and coadaptation have been observed at a broad scale (Nishiguchi *et al.*, 1998), but finer-scale genetic analyses show incongruent and low levels of genetic structuring across a geographic scale (Jones *et al.*, 2006). The question remains as to how species specificity arises given the lability of associations at finer scales. Resolving this question will require coupling studies of specificity at multiple scales (among strains, among species, and so on) with studies of the genetics underlying host–parasite adaptations.

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