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Exploiting a mutualism: parasite specialization on cultivars within the fungus-growing ant symbiosis

Nicole M. Gerardo[1,2*], Ulrich G. Mueller[1,2], Shauna L. Price[2,3] and Cameron R. Currie[2,3]

[1]*Section of Integrative Biology, Patterson Laboratories, University of Texas, Austin, TX 78712, USA*
[2]*Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Republic of Panamá*
[3]*Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045, USA*

Fungus-growing ants, their cultivated fungi and the cultivar-attacking parasite *Escovopsis* coevolve as a complex community. Higher-level phylogenetic congruence of the symbionts suggests specialized long-term associations of host–parasite clades but reveals little about parasite specificity at finer scales of species–species and genotype–genotype interactions. By coupling sequence and amplified fragment length polymorphism genotyping analyses with experimental evidence, we examine (i) the host specificity of *Escovopsis* strains infecting colonies of three closely related ant species in the genus *Cyphomyrmex*, and (ii) potential mechanisms constraining the *Escovopsis* host range. Incongruence of cultivar and ant relationships across the three focal *Cyphomyrmex* spp. allows us to test whether *Escovopsis* strains track their cultivar or the ant hosts. Phylogenetic analyses demonstrate that the *Escovopsis* phylogeny matches the cultivar phylogeny but not the ant phylogeny, indicating that the parasites are cultivar specific. Cross-infection experiments establish that ant gardens can be infected by parasite strains with which they are not typically associated in the field, but that infection is more likely when gardens are inoculated with their typical parasite strains. Thus, *Escovopsis* specialization is shaped by the parasite's ability to overcome only a narrow range of garden-specific defences, but specialization is probably additionally constrained by ecological factors, including the other symbionts (i.e. ants and their antibiotic-producing bacteria) within the coevolved fungus-growing ant symbiosis.

Keywords: fungus-growing ants; coevolution; *Escovopsis*; host–parasite interactions; Attini; parasite specificity

1. INTRODUCTION

Most parasites are host specific, specializing on particular host genotypes (Carius *et al.* 2001), on monophyletic host lineages (Herre 1993; Johnson *et al.* 2002) or on unrelated but phenotypically similar hosts (Morand *et al.* 2002; Waldenstrom *et al.* 2002). The extent of a parasite's host range affects the ecological dynamics of host–parasite systems (Woolhouse *et al.* 2001), which in turn influence long-term coevolutionary interactions. Thus, parasite specialization can lead to patterns of congruence in host and parasite phylogenies, suggesting coevolution and cospeciation of both symbionts (Clayton *et al.* 2003*a*,*b*). Such associations are known for a wide spectrum of host–parasite associations, including vertebrates and their lice (Hafner *et al.* 1994; Clayton & Johnson 2003), birds and their brood parasites (Sorenson *et al.* 2004), and cultivated fungi of attine ants and their garden parasites in the genus *Escovopsis* (Currie *et al.* 2003*b*).

Specificity arises as a consequence of a parasite's adaptation to environmental and symbiotic forces (Combes 2001). A parasite's host range may be limited by its ability (i) to persist in the habitat of particular hosts (Norton & Carpenter 1998), (ii) to recognize and locate susceptible hosts (Sorenson *et al.* 2003), or (iii) to overcome the defences of particular hosts (Van der Ackerveken & Bonas 1997). In many parasite systems, it has been possible to determine the host range of a parasite, but the mechanistic and selective processes determining parasite specificity have remained elusive.

This study elucidates the processes shaping fine-scale species-level parasite specialization in the fungus-growing ant symbiosis. The parasite *Escovopsis* is a morphologically diverse microfungal genus that attacks and consumes fungal cultivars of attine ants (Currie *et al.* 1999*a*; Reynolds & Currie 2004). *Escovopsis* is horizontally transmitted between colonies and appears to be specialized on the symbiosis; it has been found associated only with fungus-growing ant gardens and dumps (Currie *et al.* 1999*a*, 2003*b*; Currie 2001*a*). *Escovopsis* directly attacks and consumes the ants' main cultivated food source, indirectly decreasing ant-colony survival and reproduction (Currie *et al.* 1999*a*; Currie 2001*b*). Even though the ants use *Escovopsis*-specific sanitary behaviours to remove the parasite from their colonies (Currie & Stuart 2001) and have filamentous actinomycete bacteria on their exoskeletons that produce *Escovopsis*-inhibiting antibiotics (Currie *et al.* 1999*b*, 2003*a*), infections are persistent and detrimental (Currie *et al.* 1999*a*; Currie 2001*b*).

Because *Escovopsis* is harmful to both ants and their cultivars, the parasite can be hypothesized to track the evolution of either the ants, which have lower fitness in the face of garden infection, or their cultivars, which are directly attacked. For example, if cultivars can inhibit *Escovopsis*, then the parasites may infect only gardens whose defences they can overcome, leading to matching of the cultivar and

* Author for correspondence (ngerardo@mail.utexas.edu).

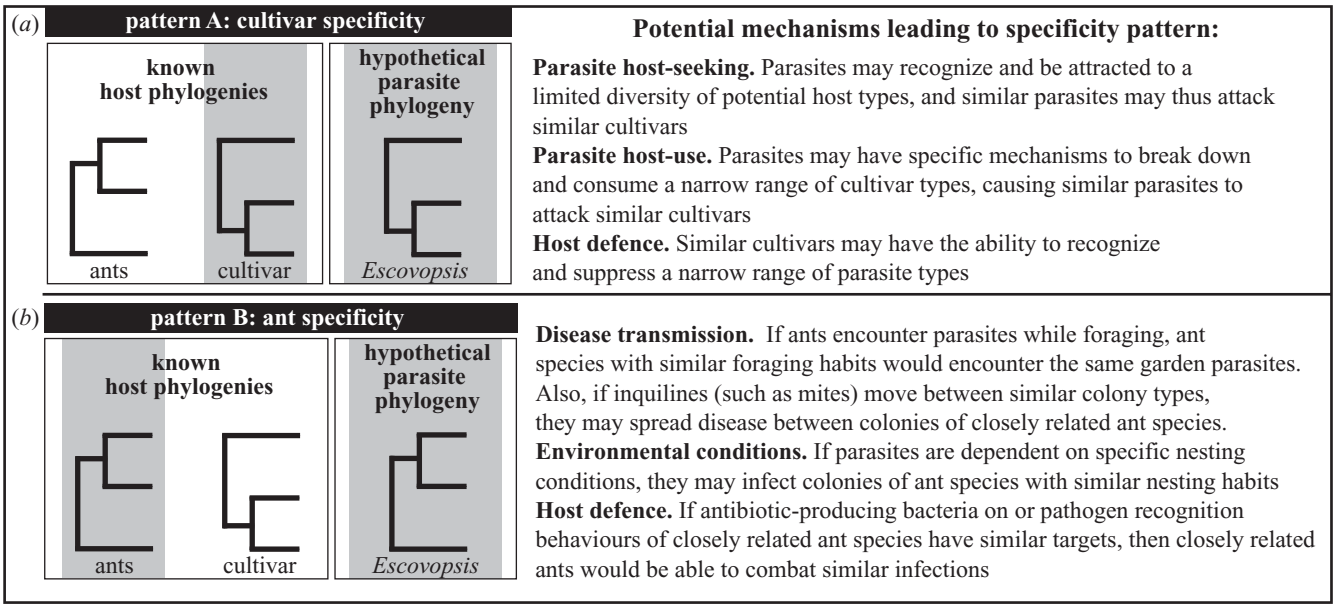

Figure 1. Topological relationships between the phylogenies predicted by two alternative hypotheses of parasite specialization. (*a*) *Escovopsis* could be specific to the cultivar that it attacks (pattern A, congruent parasite and cultivar phylogenies), or (*b*) *Escovopsis* could be specific to the ant species in whose garden it is found (pattern B, congruent parasite and ant phylogenies). Grey boxes enclose congruent host and parasite phylogenies in each case. Several mechanisms known to operate in other host–parasite systems are listed on the right, and each mechanism alone could lead to the respective pattern of specificity.

parasite phylogenies (figure 1*a*). However, if ants can recognize and weed only a limited range of *Escovopsis* strains, a particular parasite strain may infect only colonies in which it can overcome the ants' defences, leading to matching of the parasite and ant phylogenies (figure 1*b*). Alternatively, the pattern could be more complicated if it is shaped by an interplay of ant, bacterial and cultivar inhibition.

To determine whether *Escovopsis* is specialized on either particular ant or cultivar hosts, we characterized the association of *Escovopsis* with three sympatric host-ant species in the genus *Cyphomyrmex*. *Cyphomyrmex longiscapus* and *C. muelleri* are putative ant sister species with similar habits (Schultz *et al.* 2002). Both species have nests along rainforest stream banks and hillsides, with a single chamber of fungus protected by a mud auricle at the nest entrance (figure 2*a*). Despite their similarities in habit, these two closely related ant species are known to cultivate distantly related morphologically distinct fungal cultivars (Mueller *et al.* 1998; Schultz *et al.* 2002; figure 2*b*). *Cyphomyrmex costatus*, however, is a more distantly related ant species, with larger colonies found under rocks and logs that are rarely in close proximity to *C. longiscapus* and *C. muelleri* colonies. *Cyphomyrmex muelleri* and *C. costatus*, however, grow morphologically similar and occasionally genotypically identical fungal cultivars (Green *et al.* 2002; figure 2*b*), indicating that these two ant species are specialized on the same narrow clade of cultivar strains. Thus, phylogenetic patterns indicate a decoupling of ant and cultivar relationships in this system: closely related ants (*C. muelleri* and *C. longiscapus*) grow distantly related cultivars, and distantly related ants (*C. muelleri* and *C. costatus*) grow closely related or identical cultivar strains. Colonies of all three species are infected with the same pink *Escovopsis* morphotype (figure 2*c*).

Here, we analyse both amplified fragment length polymorphism (AFLP) and sequence data for *Escovopsis* isolates from *C. longiscapus*, *C. muelleri* and *C. costatus* colonies to examine patterns of association between *Escovopsis* genotypes and their hosts. We then couple these molecular analyses with cross-infection experiments to explore potential mechanisms constraining parasite host range (figure 1).

2. MATERIAL AND METHODS

(a) *Collection, natural infection rates and isolation*

We collected 118 *C. longiscapus*, 90 *C. muelleri* and 28 *C. costatus* colonies in 2001 and 2002 at six sites in the hosts' sympatric range in the Republic of Panamá: El Llano–Cartí Suitupo Road (EL), Fort Sherman (FS), Barro Colorado Island (BCI), Gamboa (GA), Ancon Hill (AH) and Pipeline Road (PLR) (see Green *et al.* (2002) for map). To determine natural infection levels in each of the three host populations, at least 10 garden pieces (*ca.* 8 mm^3) from each colony were grown on potato dextrose agar (Difco, Detroit, MI, USA) with antibiotics (50 $mg\,l^{-1}$ each of penicillin and streptomycin). If *Escovopsis* emerged from a garden piece, which typically occurred within 10 days of initial isolation, the colony was scored as infected. *Escovopsis* mycelium was then subcultured, and axenic (pure) cultures were stored at −80 °C until DNA extraction, which followed a cetyltrimethylammonium bromide (CTAB) extraction protocol modified from Bender *et al.* (1983).

(b) *Sequencing analysis*

Sequencing targeted a 1727 nucleotide stretch spanning four exons and two introns of nuclear elongation factor-1 alpha (EF-1 alpha). A single *Escovopsis* isolate from each of eight *C. longiscapus* colonies (two EL and six PLR colonies), 14 *C. muelleri* colonies (two BCI, two FS and 10 PLR colonies) and 11 *C. costatus* colonies (one BCI, one GA and nine PLR colonies) was sequenced. We also sequenced *Escovopsis* isolates from three

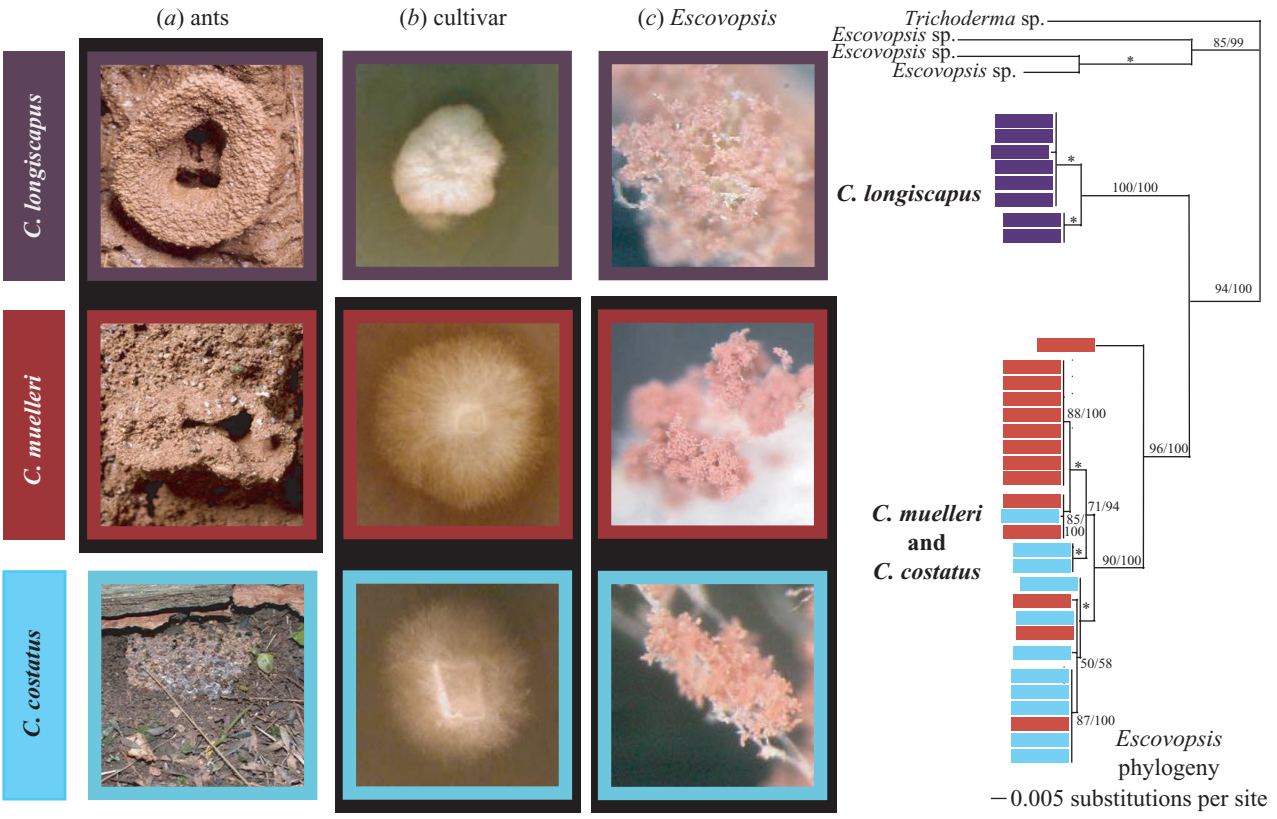

Figure 2. Relationships between the symbionts in the *Cyphomyrmex* system. (*a*) *Cyphomyrmex longiscapus* and *C. muelleri* are closely related ant species with similar nest architectures (nests in black box) whereas *C. costatus* is a more distantly related ant species with larger colonies. (*b*) *Cyphomyrmex longiscapus* grows a distantly related morphologically distinct cultivar to that of *C. muelleri* and *C. costatus*, whose cultivars (linked in black box) are morphologically and genetically similar. (*c*) *Escovopsis* isolates from all three species are morphologically similar, but EF-1 alpha sequence analysis indicates that *Escovopsis* isolates from *C. muelleri* (red) and *C. costatus* (light blue) colonies are more similar to one another than they are to *Escovopsis* isolates from *C. longiscapus* (purple) colonies. The support values are listed above the branches (likelihood support/Bayesian posterior probability) for branches with more than 50% likelihood support. An asterisk indicates branches for which both support values are greater than 95.

Apterostigma dentigerum colonies and one isolate of *Trichoderma* sp. as outgroups. Primers EF1-983F (5′-GCYCCYGGHCAYCGT GAYTTYAT-3′) and EF1-2218 (5′-ATGACACCRACRGC RACRGTYTG-3′) spanned a single exon, whereas primers EF1-3f (5′-CACGTCGACTCCGGCAAGTC-3′) and EF1-5r1 (5′-GTGATACCACGCTCACGCTC-3′) spanned three exons and two introns. Internal sequencing primers EF1-6mf (5′-GTCAC BACYGAAGTCAAGTC-3′) and EF1-6mr (5′-GACTTGAC TTCRGTVGTGAC-3′) were used for cycle sequencing in the former case. All sequences have been deposited in GenBank (accession numbers AY629361–AY629398).

Sequences were assembled in Seqman II v. 5.05 (DNASTAR), aligned using ClustalW WWW (http://www.ebi.ac.uk/clustalw) and edited manually in Macclade v. 4.06 (Maddison & Maddison 2003). The alignment was annotated based on sequences of *Gibberella circinata* (GenBank accession number AF333930) and *Gongronella butleri* (AF157252). Exon alignments were unambiguous, but intron sequences were unalignable and were excluded.

The aligned sequences were analysed in PAUP* v. 4.0b10 (Swofford 2002) using maximum likelihood (ML) and a general time reversible (GTR) sequence-evolution model with four Γ-distributed rate classes and a proportion of invariant sites (PINVAR). This model was chosen based on results from Modeltest v. 3.06 (Posada & Crandall 1998). Tree searches were conducted via tree bisection–reconnection (TBR) branch swapping on five stepwise-addition trees (assembled in random order). We estimated initial parameters on maximum-parsimony trees and then refined the parameters via successive approximation on trees recovered using maximum likelihood. These final parameters were used in all successive analyses and simulations.

We assessed support for each branch using both bootstrap and Bayesian analyses. Non-parametric bootstrap proportions were estimated from 100 pseudo-replication datasets analysed under the ML criterion. Bayesian posterior probabilities were estimated as the proportion of trees sampled after burn-in that contained each of the observed bipartitions. Bayesian analyses were performed using Mrbayes v. 3.0b4 (Huelsenbeck & Ronquist 2001) with GTR + Γ + PINVAR parameters estimated during the run, using the default value of four Markov chains and a temperature parameter set to 0.2. We combined trees after burn-in from four Monte Carlo Markov chains (500 000 generations run^{-1}, trees sampled every 100 generations, burn-in at 50 000 generations). All trees remaining after burn-in were used to construct a majority-rule consensus tree.

We used analysis of molecular variance (AMOVA) in Arlequin v. 2.001 (Schneider *et al.* 2000) to partition the sequence variation among isolates within and between host species. F_{st} values were then generated to make pairwise comparisons between populations, where each group of parasites isolated from one of the three hosts was considered a population. Levels of significance were determined through 100 000 random-permutation replicates.

A Bonferroni correction was used to correct for multiple pairwise comparisons.

(c) *Amplified fragment length polymorphism analysis*

To investigate the phylogenetic relationships within a larger collection of *Escovopsis* isolates, we analysed the relationships between 126 *Escovopsis* isolates from a total of 42 colonies, using AFLP genotyping methods (Mueller & Wolfenbarger 1999). Twenty-four of these 126 isolates were part of the above-mentioned sequencing analysis (see § 2b). We included multiple *Escovopsis* isolates from single colonies to establish whether single gardens could be infected by multiple parasite genotypes. Isolates included *Escovopsis* from 11 *C. longiscapus* colonies (two EL and nine PLR colonies; averaging 3.6 isolates colony^{-1}) 21 *C. muelleri* colonies (one EL, four BCI, four FS and 12 PLR colonies; averaging 3.5 isolates colony^{-1}) and 10 *C. costatus* colonies (two AH, one BCI, one GA and six PLR colonies; averaging 1.4 isolates colony^{-1}).

AFLP markers were generated on an ABI Prism 3100 genetic analyser and scored in GENOTYPER v. 2.5. Reactions followed the AFLP protocol for small plant genomes (http://www.appliedbiosystems.com; protocol 4303146), with the modification that preselective products were diluted 2 : 1 before use in the selective reactions. Five combinations of AFLP-primer extensions were chosen because they generated high levels of polymorphic markers that could be scored reliably: AC/CAT, TC/CAA, TG/CAA, TG/CTA and TC/CAG. AFLP markers were scored blindly by simultaneously comparing all fragments of a given length across all 126 *Escovopsis* isolates. Only markers that could be scored as unambiguously present or absent across all 126 samples were used in the analysis.

The final AFLP matrix included 299 informative characters, which were analysed in a two-step process under the parsimony criterion in PAUP*. In the first step, we completed a heuristic search without saving multiple trees (Multrees off; 50 000 replicates). We then used the best trees from this search as the starting point for a heuristic search (Maxtree of 500 000; Multrees on). Parsimony bootstrap analysis included 500 pseudoreplicates (five stepwise addition searches per pseudoreplicate; Maxtree of 100).

As with the sequence data, we also used AMOVA and comparison of between-host pairwise F_{st} values to partition AFLP variation across *Escovopsis* isolates from the three hosts. To prevent pseudoreplication, we randomly selected only one *Escovopsis* isolate per colony (total of 42 isolates) for AMOVA analysis.

(d) *Cross-infection experiments*

To determine the impacts of *Escovopsis* on typical and atypical hosts, we inoculated garden material with *Escovopsis* isolates from each of the three host types. We used garden pieces from 27 *C. longiscapus* colonies, 38 *C. muelleri* colonies and 26 *C. costatus* colonies. For each colony, we placed four garden fragments (*ca.* 100 mg fragment^{-1}) without ants onto separate sterile Petri dishes lined with moist cotton and sealed with parafilm. One garden fragment per colony was randomly assigned to each of the four treatments: (i) inoculation with *Escovopsis* from a *C. longiscapus* colony; (ii) inoculation with *Escovopsis* from a *C. muelleri* colony; (iii) inoculation with *Escovopsis* from a *C. costatus* colony; or (iv) control. We inoculated each garden piece with a small piece (*ca.* 6 mm^3) of agar with spore-bearing mycelium of an *Escovopsis* culture less than two weeks old. Pieces were cut from media at the leading edge of the fungal growth and placed in direct contact with the garden fragment. Controls were 'inoculated' with a piece of sterile agar.

For each treatment, garden pieces were randomly assigned *Escovopsis* strains originally isolated from one of three colonies of the appropriate host species. Because we used only three different *Escovopsis* strains per host, the results statistically represent the impact of these particular isolates rather than that of the population of *Escovopsis* as a whole. These isolates, however, have genotypes common to parasites found in the host populations and thus are representative of the typical parasite population (all experimental *Escovopsis* strains were confirmed via AFLP or sequence analysis to have genotypes frequently isolated from the associated host type). All experimental parasite isolates and *Cyphomyrmex* colonies were from the Panama Canal region.

Over a two-week period, we monitored garden fragments daily for *Escovopsis* growth. The level of growth was recorded as either suppression (no growth on garden) or overgrowth (*Escovopsis* grew over the entire garden). All colonies for which the control garden fragment was overgrown with *Escovopsis* were considered to have a previously established natural infection. We thus excluded all garden fragments (both treatment and control) from these previously infected colonies, leaving garden fragments from a total of 26 *C. longiscapus* colonies (4% of colonies excluded), 23 *C. muelleri* colonies (39% of colonies excluded) and 18 *C. costatus* colonies (31% of colonies excluded) for analysis. These prior infection rates parallel the frequency of infection detected in natural field conditions (see § 3).

We used the GENLOG procedure in SPSS v. 11.5.5 (SPSS Inc., Chicago, IL, USA) to determine whether there was an overall interaction between *Escovopsis* type, garden type and infection establishment. This procedure uses a χ^2 square goodness-of-fit test to determine the independence of three or more categorical variables. We also used individual two-way χ^2 square analyses to determine whether infection rate varied for a given garden type according to the treatment.

3. RESULTS

(a) *Natural field infection rates*

Escovopsis infection in *Cyphomyrmex* colonies is common. *Escovopsis* emerged in 12% of *C. longiscapus* colonies, 29% of *C. muelleri* colonies and 60% of *C. costatus* colonies. Infection rates for *C. muelleri* and *C. costatus* colonies are similar to infection rates reported for colonies of other attine genera (e.g. 33–51% across five genera in Currie *et al.* (1999*a*)), but the infection rate for *C. longiscapus* colonies is lower than that previously reported for other attines. These and previously reported values probably represent a conservative estimate of the rate of natural infection, because some infections remain undetected when only 10 garden pieces per colony are sampled (N. M. Gerardo and C. R. Currie, unpublished data).

(b) *Sequencing analysis*

Out of the 1157 positions in our final sequence alignment, 237 sites were variable and 165 of these were parsimony informative. ML analysis supported a single best tree. In this tree, *Escovopsis* isolates from *C. longiscapus* colonies formed a well-supported clade (figure 2). Isolates from *C. muelleri* and *C. costatus* colonies fell into another well-supported clade. In several instances, EF-1 alpha sequences of *Escovopsis* isolates from *C. muelleri* and *C. costatus* colonies were identical.

Table 1. AMOVA results and population pairwise comparisons based on sequence and AFLP data.
(Overall F_{st} values indicate the proportion of variation seen in (*a*) sequence data and (*b*) AFLP data that is attributable to parasite genotype differences between the three hosts. Pairwise comparisons are between *Escovopsis* isolated from host gardens of the three ant species *Cyphomyrmex longiscapus*, *C. muelleri* and *C. costatus*. All *p*-values were calculated by permuting genotypes among samples (100 000 permutations). All *p*-values for pairwise comparisons are less than 0.0001.)

	(*a*) sequence data			(*b*) AFLP data		
AMOVA results	variance	d.f.	% total	variance	d.f.	% total
between hosts	16.61	2	70.46	6.61	2	22.37
within hosts	6.96	30	29.54	22.96	39	77.63
	overall $F_{st} = 0.70$, $p < 0.01$			overall $F_{st} = 0.22$, $p < 0.01$		
between-host pairwise comparisons						
	pairwise F_{st}			pairwise F_{st}		
C. longiscapus and *C. muelleri*	0.77			0.24		
C. longiscapus and *C. costatus*	0.90			0.35		
C. muelleri and *C. costatus*	0.21			0.11		

Consistent with these results, AMOVA of 35 sequenced samples revealed that 70% of EF1-alpha sequence variation was explained by the host type from which the parasite was isolated (table 1*a*). Pairwise comparisons revealed significant differences between *Escovopsis* from each of the three host types. There was, however, a much lesser difference between *Escovopsis* from *C. costatus* and *C. muelleri* colonies than between *Escovopsis* from *C. longiscapus* and *C. muelleri* colonies or between *Escovopsis* from *C. longiscapus* and *C. costatus* colonies (table 1*a*).

Thus, the sequence data indicate that *C. longiscapus* and *C. muelleri* (closely related ants that cultivate distantly related fungi) are associated with different pathogens, whereas *C. muelleri* and *C. costatus* (more distantly related ants that grow similar fungal cultivars) are associated with similar pathogens. *Escovopsis* therefore is cultivar-type rather than ant-type specific.

(c) *Amplified fragment length polymorphism analysis*

AFLP data suggested a similar pattern of cultivar specificity. Parsimony analysis of 299 informative AFLP characters was terminated with 500 000 equally parsimonious trees. The consensus tree (figure 3) contains three main genotype clusters separated by long branches with strong parsimony bootstrap support: one clade with *Escovopsis* isolates from only *C. muelleri* colonies; a second clade primarily comprising *C. muelleri* and *C. costatus Escovopsis* isolates; and a third clade with mostly *C. longiscapus* isolates. All eight isolates from *C. longiscapus* colonies that were included in both the AFLP and sequence studies fell within the single '*longiscapus*-type' genotype cluster in the AFLP parsimony consensus tree (bottom right clade, figure 3), and all the 16 *C. muelleri* and *C. costatus* isolates included in both studies fell within a single AFLP genotype cluster (top right clade, figure 3). Thus, the AFLP study, which included more samples, revealed an entire clade of '*muelleri*-specific' *Escovopsis* (top left clade, figure 3) that was not apparent in the more sample-limited sequencing analysis.

Single *Cyphomyrmex* gardens are occasionally infected by multiple *Escovopsis* strains. In the 22 cases in which we were able to genotype multiple *Escovopsis* isolates from the same colony, there were three instances where isolates from a single colony fell into unambiguously distinct genotype clusters, indicating infection by multiple parasite genotypes. In the remaining 19 instances where multiple samples from a single garden were genotyped, the AFLP profile differences were minor (e.g. less than 3% of bands differed). Because small AFLP profile differences may be artefacts rather than actual genotypic differences, these 19 colonies were conservatively assumed to have a single infection.

AMOVA analysis of AFLP data revealed that a significant proportion of the variation (22%) was a result of between-host differences. This is lower than the amount of variation explained by between-host differences using sequence information (70%). This disparity may be because AFLP markers evolve more rapidly than sequences or because mutation in AFLP markers is likely to result in autapomorphies that would increase the extent of within-host variation. Despite this, comparison of between-host pairwise F_{st} values showed the same pattern as sequence data analysis, with more similar *Escovopsis* genotypes infecting similar cultivars (table 1*b*). Thus, both parsimony and AMOVA analyses of the AFLP data suggest that *Escovopsis* is cultivar-type specific.

AFLP analyses revealed two parasite isolates from *C. muelleri* colonies that were more similar to isolates from *C. longiscapus* colonies than they were to other *Escovopsis* from *C. muelleri* colonies. Similarly, two isolates from *C. longiscapus* colonies were more similar to isolates from *C. muelleri* and *C. costatus* colonies than they were to other *Escovopsis* from *C. longiscapus* colonies (figure 3). These isolates associated with 'atypical' hosts represent only 3% of all isolates, but they do indicate that *Escovopsis* can occasionally be associated with atypical hosts. Because we kept colonies separated from one another prior to isolation, these samples associated with atypical hosts are not likely to be a result of post-collection laboratory cross-infection, although this cannot be ruled out entirely. It is interesting to note that one of the '*longiscapus*-type' *Escovopsis* samples from a *C. muelleri* colony was isolated in a colony that was only 3 cm away from a *C. longiscapus* colony in the field, suggesting that infection may occasionally spread to neighbouring colonies even if the garden is of an atypical cultivar-host type.

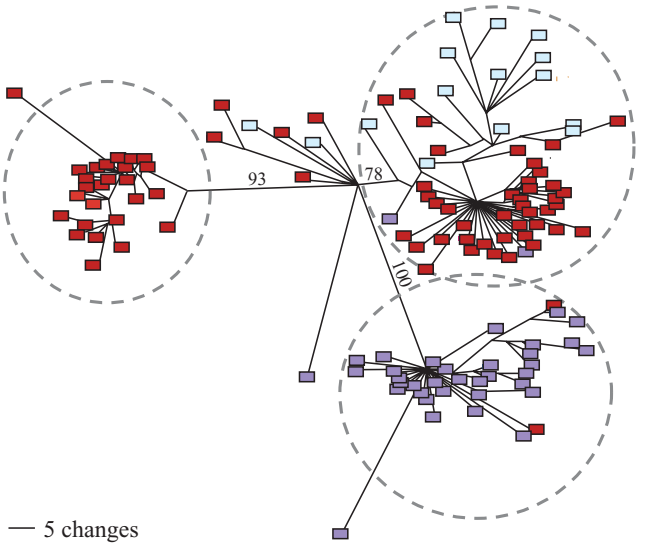

Figure 3. AFLP phylogeny of *Escovopsis* isolates from the three host species. Unrooted strict-consensus phylogram based on AFLP data generated through parsimony analysis. The support values are indicated on branches separating the three main genotype clusters (identified by dashed circles). One genotype cluster is composed of only *Escovopsis* isolates from *Cyphomyrmex muelleri* (red) colonies, a second genotype cluster is composed mostly of isolates from *C. muelleri* and *C. costatus* (light blue) colonies, and a third cluster is composed mostly of isolates from *C. longiscapus* (purple) colonies.

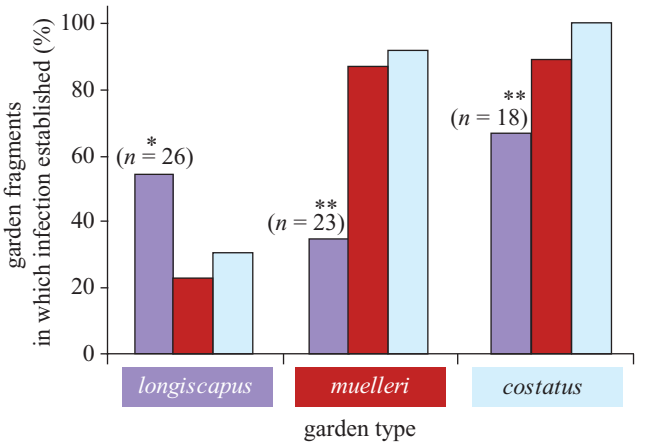

Figure 4. *Escovopsis* infection rates in cross-infection experiments. Garden pieces from presumably uninfected *Cyphomyrmex longiscapus*, *C. muelleri* and *C. costatus* colonies were inoculated with *Escovopsis* isolated from a *C. longiscapus* colony (purple), from a *C. muelleri* colony (red) or from a *C. costatus* colony (light blue). The graph indicates the percentage of pieces of a given garden type in which a particular *Escovopsis* type succeeded in establishing infection. On *C. muelleri* and *C. costatus* garden types, ** indicates that infection was significantly less likely to establish with *Escovopsis* from *C. longiscapus* colonies ($p < 0.05$). On *C. longiscapus* gardens, * indicates that *Escovopsis* from *C. longiscapus* colonies established infection more often than the other *Escovopsis* types, although this difference was not significant at the $p < 0.05$ level ($p = 0.055$).

(d) *Cross-infection experiments*

We found that *Escovopsis* strains from colonies of all three ant species could infect and overgrow garden pieces from each colony type (figure 4). Overall, infection established more frequently on *C. muelleri* and *C. costatus* garden pieces (71% and 85%, respectively) than on *C. longiscapus* garden pieces (36%), corresponding to lower levels of natural field infection in *C. longiscapus* colonies (see § 3a).

Significant differences in infection establishment are evident across the three colony types (figure 4). A χ^2 goodness-of-fit analysis indicated a significant interaction between garden type, *Escovopsis* type and infection establishment (Pearson $\chi^2 = 30.56$, d.f. $= 4$, $p < 0.0001$). Analysing infection in each garden type separately, there was a significant interaction between *Escovopsis*-type and infection establishment on both *C. muelleri* (Pearson's $\chi^2 = 22.11$, d.f. $= 2$, $p < 0.0001$) and *C. costatus* (Pearson's χ^2 with Yate's continuity correction $= 8.2174$, d.f. $= 2$, $p = 0.016$) gardens. For both *C. muelleri* and *C. costatus* gardens, infection establishment was equally likely when inoculated with *Escovopsis* isolates from *C. muelleri* and *C. costatus* colonies (for *C. muelleri* gardens: $\chi^2 = 0.22$, d.f. $= 1$, $p = 0.64$; for *C. costatus* gardens: $\chi^2 = 0.53$, d.f. $= 1$, $p = 0.47$) but was significantly less frequent when inoculated with *Escovopsis* from *C. longiscapus* colonies (for *C. muelleri* gardens: $\chi^2 = 19.44$, d.f. $= 1$, $p < 0.0001$; for *C. costatus* gardens: $\chi^2 = 5.30$, d.f. $= 1$, $p = 0.02$). For *C. longiscapus* colonies, a similar host-specific pattern emerged, where infection established more frequently when *C. longiscapus* gardens were inoculated with *Escovopsis* isolates from *C. longiscapus* colonies than from either *C. muelleri* or *C. costatus* colonies, though this result was not statistically significant at the $p = 0.05$ level (Pearson $\chi^2 = 5.794$, d.f. $= 2$, $p = 0.055$).

4. DISCUSSION

The garden parasite *Escovopsis* is host specific, tracking the cultivar in the *Cyphomyrmex* fungus-growing ant system. We found that genotypically similar parasites attack the similar cultivars raised by *C. muelleri* and *C. costatus*, whereas more genotypically distant parasites attack the cultivar raised by *C. longiscapus*. In cross-infection experiments, *Escovopsis* strains were more likely to establish infection on typical than on atypical fungal-host species, providing further evidence for host-species specificity.

Moreover, the congruence of cultivar and parasite phylogenetic relationships suggests possible further within-host specificity. Although *Escovopsis* of *C. muelleri* and *C. costatus* are more genetically and phenotypically similar to each other than to *Escovopsis* attacking *C. longiscapus* colonies, and although *C. muelleri* and *C. costatus* colonies are sometimes infected with identical *Escovopsis* strains, AMOVA did reveal significant differences between *Escovopsis* attacking *C. muelleri* and *C. costatus* colonies. Likewise, Green *et al.* (2002) showed that *C. muelleri* and *C. costatus* cultivars are occasionally genotypically identical; however, some cultivar strains are associated with only one of the two ant hosts. Analogous cultivar and *Escovopsis* population structures suggest that the parasite may closely track within-species host genotypes, possibly in a coevolutionary arms race. Future analyses of cultivars and parasites isolated from the same colonies will determine the extent of parasite host-genotype specificity in the attine system.

What dictates *Escovopsis* specificity? Although many parasites are habitat-restricted, either because they themselves can survive only in certain niches or because their vectors function only within certain niches (Norton & Carpenter 1998; Jaenike & Perlman 2002), such habitat specialization does not seem to be the case for *Escovopsis* in the *Cyphomyrmex* system. *Cyphomyrmex longiscapus* and *C. muelleri* colonies are found in similar habitats, are often located within centimetres of each other in the field and have nearly the same garden architecture and size (figure 2*a*; Schultz *et al.* 2002). However, despite their close spatial proximity and relatively open nest architectures, *C. longiscapus* and *C. muelleri* colonies are consistently infected by different *Escovopsis* strains, suggesting that habitat does not constrain *Escovopsis*–host associations. If vector biology maintains *Escovopsis* specificity, the vector itself would have to be cultivar specific rather than habitat specific. Although vector-driven specificity seems somewhat unlikely in the *Cyphomyrmex* system, it is a possibility, and further natural-history observations and experimentation are needed to determine the mechanism by which *Escovopsis* is horizontally transmitted.

Instead, *Escovopsis* specificity is probably a result of parasite and host adaptation. For example, parasites may be adapted to locate and use the resources of particular hosts efficiently. *Escovopsis* is attracted to chemical signals produced by host cultivars (N. M. Gerardo and C. R. Currie, unpublished data). This attraction may allow *Escovopsis* efficiently to establish and maintain infection as it effectively moves through the garden matrix and finds cultivars for consumption. If *Escovopsis* is adapted to recognize chemical signals produced by specific cultivar types, host-seeking may limit *Escovopsis* to utilizing a narrow range of chemically similar cultivars. However, when experimentally forced into contact with cultivars from all three *Cyphomyrmex* hosts, *Escovopsis* strains were often unable to infect garden pieces, particularly of atypical hosts. This suggests that even if *Escovopsis* could efficiently seek a wide range of hosts, it might not be able to exploit all of them. This may be because *Escovopsis* is adapted to use only certain hosts as a nutritional resource. However, *Escovopsis* strains isolated from all three host types could sometimes successfully infect all three garden types, demonstrating that certain *Escovopsis* isolates were able to consume all host garden types. Parasite host seeking and host use (figure 1) are therefore probably coupled with other factors, such as host defence, in maintaining *Escovopsis* specificity.

When potentially virulent infections are common, hosts are selected to adapt defences targeted against their parasites, and parasites are then selected to overcome their host's novel defences. This perpetual race to adapt is a central theme in host–parasite biology and modern medical evolutionary genetics. In the *Cyphomyrmex* system, we see that natural infection is common, and *Escovopsis* has previously been shown to decrease colony fitness and survival (Currie *et al.* 1999*a*; Currie 2001*b*). Thus, tightly coupled host–parasite coevolution is expected. Consistent with this expectation, infection was more likely to establish in cross-infection experiments when hosts were inoculated with parasites isolated from a closely related host than from a distantly related host, suggesting that *Escovopsis* strains are adapted to overcome the defences of a limited range of host gardens. Because these gardens are a complex matrix composed of cultivar, soil fungi, endophytic fungi, antibiotic compounds produced by ants, forage material and possibly even remnants of the actinomycete bacteria from the ants' cuticles, further work is needed to determine the precise mechanism by which the host garden defends itself against *Escovopsis* attack.

None of the three experimentally infected host types could defend against all atypical parasite strains. This may explain the rare atypical infections seen in nature, where 3% of colonies were infected by a parasite strain with which that host is not normally associated (figure 3).These atypical natural infections may be owing to differential host susceptibility, and the likelihood of atypical infection may increase when the host is already infected with another parasite. All of these atypical infections were in colonies infected with other typical strains, suggesting that, as previously hypothesized (May & Nowak 1995; Read & Taylor 2001), host susceptibility may be affected by the presence of multiple parasites. Further work examining host–parasite genotype interactions and multiple infection dynamics may determine under what circumstances such atypical infections are able to establish and persist.

Interestingly, *C. longiscapus* gardens were less susceptible to experimental infection and had lower natural infection rates than those of *C. muelleri* and *C. costatus*, suggesting that some component of the garden matrix is better adapted to inhibiting *Escovopsis* in *C. longiscapus* than in *C. muelleri* and *C. costatus* colonies. The question then arises as to why *C. longiscapus* gardens might maintain higher resistance. Potential explanations include: (i) *Escovopsis* specialized on *C. longiscapus* are more virulent and thus exert greater selective pressure to maintain resistance in cultivars; (ii) *C. muelleri* and *C. costatus* gardens are released from maintaining high resistance because of other effective colony defences (e.g. actinomycete defences; see below); or (iii) the three cultivar hosts are simply at different stages of the host–parasite arms-race cycle.

What other colony defences could mediate parasite host range? The ants are known to weed and groom *Escovopsis*-infected gardens, contributing to disease suppression (Currie & Stuart 2001). If these ant behaviours are *Escovopsis*-type specific, they could influence the *Escovopsis* host range. Additionally, coevolution between actinomycete-produced antibiotics known to suppress specifically *Escovopsis* and antibiotic resistance in *Escovopsis* could play a critical role in shaping *Escovopsis* specificity. Further work is needed to test for behaviour- and antibiotic-driven coevolution. Such complexity highlights the novelty of this system, in which three mutualistic symbionts (ants, cultivar and actinomycete bacteria) are all negatively affected by the same ubiquitous parasite and thus are expected to coevolve adaptations simultaneously to combat *Escovopsis*. The ease with which these symbionts can be experimentally manipulated and genotyped makes the fungus-growing ant–microbe system ideal for future experimental work on ecological and evolutionary host–parasite dynamics.

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