Correlation between virulence and genetic structure of *Escovopsis* strains from leaf-cutting ant colonies in Costa Rica

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Leaf-cutting ants (genera *Atta* and *Acromyrmex*) cultivate a specialized fungus for food in underground chambers employing cut plant material as substrate. Parasitism occurs in this agricultural system and plays an important role in colony fitness. The microfungi *Escovopsis*, a specialized mycoparasite of the fungal cultivar, is highly prevalent among colonies. In this study, we tested the antagonistic activity of several *Escovopsis* strains from different geographical areas in Costa Rica. We employed a combination of laboratory tests to evaluate virulence, including pure culture challenges, toxicity to fungus garden pieces and subcolony bioassays. We also performed a phylogenetic analysis of these strains in order to correlate their virulence with the genetic structure of this population. The bioassays yielded results consistent between each other and showed significant differences in antagonistic activity among the parasites evaluated. However, no significant differences were found when comparing the results of the bioassays according to the source of the ants’ fungal cultivar. The phylogenetic analyses were consistent with these results: whilst the fungal cultivar phylogeny showed a single clade with limited molecular variation, the *Escovopsis* phylogeny yielded several clades with the most virulent isolates grouping in the same well-supported clade. These results indicate that there are *Escovopsis* strains better suited to establish their antagonistic effect, whilst the genetic homogeneity of the fungal cultivars limits their ability to modulate *Escovopsis* antagonism. These findings should be taken into consideration when evaluating the potential of *Escovopsis* isolates as biocontrol agents for this important agricultural pest in the Neotropics.

INTRODUCTION

The tribe Attini (subfamily Myrmicinae) is a monophyletic group of fungus-growing ants consisting of 13 genera and 230 species distributed from the southern United States to Argentina (Hölldobler & Wilson, 1990). The agricultural system of these social insects originated ~50 million years ago (Wilson, 1971). The most derived genera, *Atta* and *Acromyrmex*, use fresh plant material obtained from leaf cutting as the substrate for their fungal symbiont, *Leucoagaricus gongylophorus* (Möller, 1893; Chapela *et al.*, 1994). This basidiomycete fungus is cultured in underground nests consisting of chambers connected by tunnels, in which the fungus garden is carefully tended by the ants (Stahel, 1943). Tasks in *Atta* colonies are distributed according to the ants’ size and age, and include three well-differentiated castes: major (also called soldiers), medium (foragers) and minimum (gardeners) workers (Wilson, 1980). Their nests can occupy large areas, survive for more than 10 years and support a population of millions of ants per colony (Weber, 1966, 1972).

Given their social structure and efficiency, *Atta* colonies are one of the predominant herbivores in the Neotropics and are considered important agricultural pests in these areas (Hölldobler & Wilson, 1990). They exhibit a rapid growth rate and their combined foraging activity causes an important defoliation, consuming hundreds of kilograms of...
leaves per year (Wirth et al., 2003), and causing the destruction of plantations and gardens in tropical areas (Cherrett, 1968, 1986; Weber, 1979). Furthermore, the continuous deforestation of the land surrounding agricultural plantations increases their foraging efficiency, therefore aggravating the economic impact of these ants on Central and South American farmers (Urbas et al., 2007).

Costa Rica is no exception to the negative impact that leaf-cutting ants can have in agriculture. The most common species, Atta cephalotes, is ubiquitous <2000 m above sea level and thus affects a wide variety of crops throughout the country, including coffee, ornamentals, wood-producing and citrus trees, among others (Varon, 2006). The other Atta species, Atta colombica, has a patchy distribution and it is abundant only in the southern Pacific lowlands, where it can also attain high densities and affect agricultural operations (Longino, 2004). Although Acromyrmex species cultivate the same strain of mutualistic fungus and share many common behaviours with Atta, their colony sizes are significantly smaller (Wilson, 1980). However, common species in Costa Rica, such as Acromyrmex echinatior, which prefers open dry habitats in urban areas or seasonally dry habitats of Guanacaste Province (Longino, 2004), can also be a problem for small farmers and house gardens.

In order to effectively control these ants, it is crucial to understand their complex symbiotic associations with micro-organisms that contribute to their ecological success. As a classic example of mutualism, attine ants promote hyphal growth of their cultivar, providing a means of dispersion and protection against parasites and competitors, whilst the cultivar represents a vital nutritional source for colony development (Currie, 2001a). Other mutualistic micro-organisms described in this system include antibiotic-producing actinomycetes for protection against potential plagues (Currie et al., 1999b) and N2-fixing bacteria that complement the ants’ nutritional budget (Pinto-Tomás et al., 2009). However, parasitism also occurs in attine ant agriculture and plays an important role in colony fitness. The microfungus Escovopsis is highly prevalent in leaf-cutting ant fungus gardens and corresponds to a specialized mycoparasite of the fungal cultivar (Currie et al., 1999b).

Escovopsis (order Hypocreales) is an anamorphic ascomycete with an ancient origin in attine ant–microbe symbiosis, coevolving with these social insects and their fungal mutualist for more than 50 million years (Currie et al., 2003). This ancient origin is supported by several facts. (i) Escovopsis is a monophyletic group associated with the entire attine ant clade, including Atta and Acromyrmex. (ii) The comparison of Escovopsis phylogeny with that of attine ants and their fungal cultivars indicates that, at the deepest phylogenetic levels, the evolution of the Escovopsis parasites parallels the evolution of both the ants and the cultivars (Chapela et al., 1994; Mueller et al., 1998; Currie et al., 2003). (iii) Escovopsis is phylogenetically and morphologically diverse, suggesting a long evolutionary history (Currie et al., 2003; Currie, 2001a). (iv) No correlation was found between Escovopsis phylogeny and geographical distribution, indicating lineage mixing across large geographical areas over extensive time periods (Currie et al., 2003).

Regarding its potential as a parasite, Escovopsis is a contact necrotroph that secretes compounds to destroy the host hyphae and uses the dead biomass as a nutritional source (Reynolds & Currie, 2004). This pathogen has evolved to evade the ants’ hygienic behaviour and represents an important plague of their agricultural system given its ability to destroy fungus gardens completely. Escovopsis grows profusely on susceptible hosts, causing the collapse of the feeding structures (gongylidia), and leading to colony inanition and consequently death (Currie et al., 1999a, 2001b). Therefore, this fungus represents an important candidate as a bio-control agent for leaf-cutting ants because it is a specialized pathogen that specifically antagonizes the symbiosis between the ants and their fungal cultivar (Currie, 2001b). A highly virulent Escovopsis may establish a persistent infection in the colony, impairing the ants’ nutritional source without harming other species or affecting other ecosystems (Currie, 2001b).

To effectively employ Escovopsis as a biocontrol agent it is necessary to evaluate the antagonistic activity of different isolates against the fungal cultivar because the detrimental impact on colony survival will depend, at least to some extent, on the challenging parasite strain (Currie, 2001b; Silva et al., 2006). The mechanisms of Escovopsis pathogenicity have not been fully described; however, it is possible to evaluate its virulence through laboratory bioassays in order to identify the most aggressive strains (Taerum et al., 2007). It is also important to perform a phylogenetic analysis of both evaluated fungi to further understand the complex association between Escovopsis and L. gongylophorus, and how different strains interact with each other (Taerum et al., 2007; Gerardo et al., 2004, 2006a, b). For example, cross-infection experiments with colonies from the attine genus Cyphomyrmex showed 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 fungus gardens are inoculated with their typical parasite strains (Gerardo et al., 2004). Similarly, clades of closely related Escovopsis attack specific cultivar groups of the attine genus Apterostigma, and the cultivar and Escovopsis phylogenies match at some scales, with occasional host-switching being responsible for the discordance in host–parasite phylogenies (Gerardo et al., 2006a, b). Similar results have been obtained with Escovopsis isolated from leaf-cutting ants, where the molecular phylogeny of the parasites suggests specificity at the broad phylogenetic level, but reveals frequent host-switching events between species and genera (Taerum et al., 2010). In the present study, we focused on Atta cephalotes, one of the major insect pests in Costa Rica, to test the hypothesis that there were significant differences in the antagonistic effect of diverse Escovopsis isolates from Costa Rica against the fungal cultivar L. gongylophorus, and that these differences could be correlated with the genetic structure of both populations, therefore providing relevant
information in our quest to use Escovopsis as a biocontrol agent for this important agricultural problem.

METHODS

Isolation of Escovopsis and L. gongylophorus. Leaf-cutting ant colonies were collected in several areas of Costa Rica during 2009 and 2010, including Turrialba (Cartago province), Upala (Alajuela Province), Sarapiquí (Heredia Province) and Osa (Puntarenas Province). Species of leaf-cutting ants collected include Atta cephalotes, Atta colombica, Acromyrmex echinatior and Acromyrmex octospinus. Colonies from the attine genera Apterostigma and Trachymyrmex were also collected in Sarapiquí. These geographical regions were selected in order to represent different areas with different climate conditions in Costa Rica whose agricultural production is affected by leaf-cutting ants. The ant colonies were picked and sampled as they were sighted in the field, following their leaf-cutting activity or locating their typical nest structure. Incipient nests (1–2 years after foundation) were collected preferably due to a higher possibility of finding the queen of the colony. During collection, fungus gardens were extracted from underground chambers, aseptically transferred to sterile plastic containers with moistened cotton and transported to our laboratory at the University of Costa Rica (San Jose province). Colonies were fed three times per week from fresh leaves from local Tecoma stans trees (vainillo, Bignoniaceae).

For Escovopsis and L. gongylophorus isolation, at least 30 fungus garden pieces per colony were placed in potato dextrose agar (PDA) containing antibiotic (100 mg ampicillin L⁻¹) and incubated at room temperature. Once the fungi emerged from the garden piece, they were subcultured onto fresh media and allowed to grow. Pure cultures were stored in glycerol stocks at −80 °C. Microbial isolations were made at the collecting sites or at our laboratory, after the ants stabilized their colonies and performed their hygienic activity. They were also made prior to colony feeding in order to avoid contamination or lateral transfer of microorganisms (data on microbial isolations are given in Tables S1 and S2, available in the online Supplementary Material).

Bioassays. In order to measure Escovopsis virulence, we developed in vitro assays that represent confrontations between the parasite and the cultivar under different scenarios. In combination, these three different bioassays allowed us to identify the most virulent strains, whilst comparing the results and variability among them. In the first bioassay, we measured Escovopsis growth towards its host in pure culture within a PDA plate, placing both fungi in opposite ends and determining the distance reached by Escovopsis hyphae at a fixed time. In the second bioassay, we confronted Escovopsis with its host embedded in its natural habitat (a fungus garden piece) placed inside a moistened Petri dish and measured Escovopsis growth over the degraded substrate over time. The third bioassay consisted of a more complex design which included medium and minor workers and a larger mass of fungus garden placed in a sterile plastic container. In this case, Escovopsis was inoculated on the fungus garden and its gradual growth was measured over time. See Table S3 for details of Escovopsis isolates employed in the different bioassays.

Pure culture bioassay. Pure culture bioassays were established in diluted 1:10 PDA plates containing antibiotic (100 mg ampicillin L⁻¹). L. gongylophorus isolates were cultured individually 1 cm from the edge of each plate and incubated at room temperature for 3 weeks. Spore suspensions of 30 Escovopsis isolates were prepared individually at a concentration of 1 x 10⁶ spores ml⁻¹ and 10 µl of each suspension was inoculated 5 cm from the corresponding L. gongylophorus isolate. The concentration of the suspension was optimized by performing several preliminary tests, considering adequate amount of biomass, consistency for replicates and optimal spore versus garden/cultivar biomass ratio. Each Escovopsis isolate was tested against cultivars from three different geographical zones (Turrialba, Upala and Sarapiquí; Osa was not included due to lack of material). Negative controls were sterile distilled water (control A), a suspension of autoclaved Escovopsis spores (control B) and a similar spore suspension from an unrelated fungus (Aspergillus control C). Plates were observed over 10 days, measuring the mycelial growth of Escovopsis towards the cultivar daily.

Fungus garden piece bioassay. Healthy leaf-cutting ant colonies from Turrialba, Upala and Sarapiquí were chosen for this bioassay (colonies from Osa could not be included due to lack of material). Fungus garden pieces (1 cm³) from each colony were placed at the centre of sterile Petri plates containing cotton moistened with distilled water along the edge. All ants were removed from each piece except for two minor workers. Plates were maintained in the laboratory in the dark for 3 days to let the garden pieces stabilize and to observe any existing contamination (Currie et al., 1999a; Currie & Stuart, 2001). Aliquots of 100 µl suspensions (1 x 10⁶ spores ml⁻¹) from 30 Escovopsis isolates were inoculated over the fungus garden pieces. Each Escovopsis isolate was inoculated individually in three different plates, representing fungus garden pieces from colonies of the three selected collection areas. Negative controls were the same as in the previous assay. Plates were observed over 10 days and virulence categories were assigned each day according to Escovopsis growth over the fungus garden piece (Table 1).

Subcolony bioassay. Leaf-cutting ant colonies with healthy fungus gardens and no previous infection by Escovopsis were chosen for this bioassay. Portions (2 g) of each fungus garden were placed in clean plastic boxes to form subcolonies. To maintain a similar and proportional number of ants per subcolony, all major and medium workers were removed, and 50 medium workers were reintroduced to each subcolony; no minor workers were removed. Subcolonies were maintained in the laboratory for 3 days to allow the ants to stabilize their fungus gardens. The most virulent Escovopsis strains defined by the plate bioassays were employed in the subcolony bioassay. Spore suspensions of these strains were prepared at a concentration of 1 x 10⁶ spores ml⁻¹, and each suspension was inoculated over the surface of the corresponding fungus garden by pipetting 20 µl droplets.

<table>
<thead>
<tr>
<th>Category</th>
<th>Description of Escovopsis growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Healthy fungus garden piece, no evidence of Escovopsis mycelium and/or spores</td>
</tr>
<tr>
<td>1</td>
<td>Fungus garden piece overgrown with Escovopsis mycelium</td>
</tr>
<tr>
<td>2</td>
<td>Escovopsis mycelium and white spores overgrowing the fungus garden piece</td>
</tr>
<tr>
<td>3</td>
<td>Escovopsis mycelium, white spores and light brown spores overgrowing the fungus garden piece</td>
</tr>
<tr>
<td>4</td>
<td>Escovopsis mycelium and dark brown spores overgrowing the fungus garden piece; total collapse of the structure</td>
</tr>
</tbody>
</table>

Table 1. Virulence categories assigned according to Escovopsis growth in the fungus garden piece bioassay

See text for bioassay details.
Fig. 1. (a) Mycelial growth of Escovopsis isolates from inoculum towards its host on day 7 post-inoculation in the pure culture bioassay. Escovopsis strains belonging to a particular clade in the phylogenetic tree are marked with darker grey. C-A, control A; C-B, control B; C-C, control C.

(b) Virulence category assigned according to Escovopsis growth over the cultivar at day 7 of the fungus garden piece bioassay.
and uniformly distributing them until a final volume of 1000 μl was reached. Each Escovopsis strain was tested against two subcolonies. Negative controls were sterile distilled water, a suspension of Escovopsis autoclaved spores and a non-inoculated subcolony. The bioassay was observed over 10 days and virulence categories were assigned each day according to Escovopsis growth over the fungus garden (Table 1).

Statistical analyses. All statistical analyses for all three bioassays were performed employing the data recorded at day 7 using an ANOVA test with the statistical software Minitab 15.

DNA extraction, amplification, sequencing and phylogenetic analyses. DNA extraction from 30 Escovopsis and 15 L. gongylophorus isolates was performed following a cetyltrimethylammonium bromide protocol modified from Bender et al. (1983). Amplifications targeted the nuclear elongation factor EF-1α, using PCR primers EF1-983F and EF1-2218 according to previous phylogenetic studies on Escovopsis (Currie et al., 2003; Taerum et al., 2007, 2010; Gerardo et al., 2004, 2006b) and L. gongylophorus (Currie et al., 2003; Poulsen et al., 2009). PCR amplifications were performed with 12.5 μl EconoTaq PLUS GREEN 2X Master Mix (Lucigen), 1 μl primer EF1-2218, 1 μl primer EF1-983F and 1.25 μl template DNA, and the volume was adjusted at 25 μl with sterile water. The reaction consisted of an initialization step at 95 °C during 4 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 51 °C for 50 s and elongation at 72 °C for 2 min, and a final elongation step at 72 °C for 5 min. PCR products were sequenced either at Macrogen or at the Biotechnology Center DNA Sequencing Facility at the University of Wisconsin-Madison. Nucleotide sequences were edited manually with MEGA5 (Tamura et al., 2011) and compared with the National Center for Biotechnology Information database using BLAST (Altschul et al., 1990) in order to choose homologous sequences for phylogenetic analyses. Escovopsis sequences have been deposited in GenBank under accession numbers KF667051–KF667078 and L. gongylophorus sequences have been deposited in GenBank under accession numbers KF683203–KF683215 (Table S6).

Sequences were aligned using GUIDANCE (Penn et al., 2010), employing a MAFFT algorithm and 100 bootstrap repeats. Final editing of both Escovopsis and L. gongylophorus alignments was done in MEGA5, resulting in sequences of 858 bp for Escovopsis and 656 bp for L. gongylophorus. Phylogenetic analyses were performed through Bayesian inference, using MrBayes 3.2 (Ronquist & Huelsenbeck, 2003). A model of sequence evolution was estimated for each dataset using jModelTest (Posada, 2008). The chosen model for Escovopsis sequences was K80 (Kimura two-parameter) and for L. gongylophorus sequences was GTR (General Time Reverse). All analyses employed one cold chain and three incrementally heated chains, and a temperature parameter set to 0.1. Four separate Markov Chain Monte Carlo runs were performed, with 2 million generations each, discarding the initial 500 000 generations from each run as burn-in and sampling one in every 100 generations to calculate posterior probabilities for each branch. All trees remaining after burn-in were used to construct a majority rule consensus tree. Final editing of each phylogenetic tree was done in MEGA5 and Adobe Illustrator CS5.1. The final phylogenetic trees included 28 Escovopsis and 13 L. gongylophorus isolates.

RESULTS

Isolation of Escovopsis and L. gongylophorus

More than 70 leaf-cutting ant colonies were collected in the different areas in Costa Rica during this study. In total,
Fig. 3. (a) Escovopsis and (b) L. gongylophorus phylogenetic trees. Each taxon name indicates the isolation code, the ant species of the colony of origin and the geographical origin of the colony; the numbers in the tree branches correspond to the posterior probabilities calculated by the Bayesian method. The 10 most aggressive Escovopsis strains identified in this study.
37 Escovopsis and 19 L. gongylophorus were successfully isolated from these colonies. The parasite showed a prevalence of near 50% in this study and was easier to isolate than the cultivar given its faster growth, minimizing the risk of contamination. Also, spore separation was easier to carry out once Escovopsis emerged from the inoculum, explaining why we obtained a higher number of isolates than the cultivar itself. From these isolates, 30 Escovopsis and 15 L. gongylophorus were chosen for bioassays and phylogenetic analyses (Tables S1 and S2). These isolates represent a sample of local populations in agricultural areas affected by the foraging activities of leaf-cutting ants.

Pure culture bioassay

In this assay, Escovopsis grew towards its host, extending its hyphae considerably faster and more prominently than when growing in pure culture. Once the parasite reached its host, it inhibited its radial growth and degraded its biomass, reducing colony size and causing a change in colour (from white to yellow, see Fig. S1). However, some strains were unable to reach their host even at day 10 post-inoculation. In total, six out of 30 isolates were able to overgrow their host in pure culture at day 7 (20%).

Data on the length of Escovopsis mycelia growing towards the cultivar were recorded every day for 10 days; however, to facilitate the statistical analyses, we used the data obtained on day 7 to evaluate the behaviour of the different parasite strains as well as the impact of L. gongylophorus geographical origin on Escovopsis virulence. The latter analysis yielded no significant results ($P>0.05$), suggesting that the origin of the basidiomycete does not play an important role in its susceptibility or resistance against the parasite in pure culture. Also, Escovopsis strains did not show any enhanced growth towards a host from a specific area. However, there were significant differences in mycelial growth towards its host among the different Escovopsis strains evaluated ($P<0.05$), suggesting that some of them are more virulent than others when confronted with their host in pure culture (Fig. 1a; for detailed information on the daily progress of this bioassay, see Table S4).

Fungus garden piece bioassay

This bioassay evaluated the ability of Escovopsis to overgrow its host as it occurs in nature, being part of a fungus garden. It also allowed us to evaluate the gradual degradation of the fungus garden caused by each strain. As the parasite grew, the fungus garden piece shrank, shrivelled, turned brown and all mycelium of the basidiomycete over the degraded substrate was consumed (Fig. S2). However, not all isolates were able to efficiently consume the fungus garden, as 14 of the 30 Escovopsis isolates evaluated (47%) did not grow over any of the three fungus garden pieces from different locations. However, five out of 30 Escovopsis isolates (16.6%) were able to degrade all three of the cultivars that were presented to them at day 7.

Here, we measured Escovopsis virulence employing virulence categories according to the parasite’s growth over the fungus garden piece (Table 1). We employed the virulence categories recorded on day 7 to perform the corresponding statistical analyses and found that the geographical origin of the fungus garden piece did not cause any significant differences in the results obtained ($P>0.05$). However, there were significant differences in the virulence of the various Escovopsis isolates evaluated ($P<0.05$), further establishing that the antagonistic ability varies among different strains (Fig. 1b; for detailed information on the daily progress of this bioassay, see Table S5).

Subcolony bioassay

This bioassay was the most representative of the in vivo system, as it involved medium and minor workers. Also, a larger mass of fungus garden containing brood and leaves was available for harvesting by the ants. When the parasite completely overgrew the fungus garden, the whole structure was degraded, leading to the collapse of the subcolony (Fig. S3). As this bioassay was more labour intensive, we employed only strains that yielded the higher antagonistic activity in the previous two assays. However, out of 10 Escovopsis strains evaluated, only seven were able to collapse both subcolonies infected. Some strains only caused minor damage, being unable to affect the entire fungus garden as the infection was controlled by the ants.

The virulence categories employed in this bioassay were the same as described in the previous bioassay (Table 1), where the growth of the parasite over the fungus garden was directly proportional to the degradation of the structure. To perform statistical analyses, we employed the virulence data recorded on day 7 and again found significant differences in the virulence of the 10 isolates tested in this bioassay ($P<0.05$). Escovopsis isolates AP090225-01 and AP100526-01 proved to be more efficient when attacking leaf-cutting ant subcolonies, due to their rapid growth and quick spread over the fungus garden surface. Taking all results together, these two isolates were the most virulent parasites identified in this study, followed by isolates RM090730-02, LD100306-01, AP090731-01 and DE090731-01 (Fig. 2).

Phylogenetic analyses

According to the analysis performed, the Escovopsis isolates evaluated in this study belonged to two well-defined clades (Fig. 3a). One of them includes most isolates while the other clade was composed of seven isolates. Escovopsis
previously isolated by other researchers from Atta and Acromyrmex colonies in South America did not group with any of these two clades. No grouping according to geographical origin was observed; however, the top five most virulent Escovopsis isolates identified in this study were all grouped in the same clade (Fig. 3a).

Regarding the phylogenetic analysis of L. gongylophorus isolates, all the sequences generated in the present study were grouped in one clade (Fig. 3b). Therefore, the fungal cultivar exhibited a homogeneous genetic structure regardless of its host genera (Atta or Acromyrmex) and its geographical origin. These results are compatible with the biology of the fungal cultivar, which is dependent on clonal propagation by the ants. These results describing the genetic structure of Escovopsis and L. gongylophorus populations from each region are shown in Tables 2 and 3.

DISCUSSION

Leaf-cutting ants constitute one of the main agricultural pests in the New World Tropical regions, causing important economic losses and environmental damage due to the high amount of potent pesticides employed in their control. Due to its specificity and ability to consume the fungus gardens cultivated by the ants, the mycoparasitic ascomycete Escovopsis is an excellent candidate for the biological control of these insects. To achieve this goal, it is imperative to understand the complex relationship between the ants, their cultivar and their coevolved parasite. Previous investigations employing attine ants have demonstrated substantial differences in Escovopsis virulence and L. gongylophorus susceptibility (Currie, 2001b; Gerardo et al., 2004), and a successful infection is influenced by the combination of both symbionts’ genotypes (Gerardo et al., 2006a). Here, we focused on Escovopsis isolates from Atta and Acromyrmex from different geographical zones in Costa Rica, and evaluated their virulence using a combination of three different bioassays. These results were then correlated with the genetic structure of both the parasite and its host L. gongylophorus to further investigate the factors that govern Escovopsis virulence and to help us to select specific strains as potential candidates to develop a biocounter strategy.

The pure culture bioassay employs diluted culture media to facilitate L. gongylophorus consumption by Escovopsis by allowing mycelial growth towards its host in search of an energy source (Reynolds & Currie, 2004). This bioassay evaluates the antagonistic effect of Escovopsis against the cultivar in the absence of the ants and any other symbiotic micro-organism, including antibiotic-producing bacteria (Silva et al., 2006). Therefore, the only defence mechanism available in this setting is the release of protective compounds by the basidiomycete (Gerardo et al., 2004). Our results demonstrated that several Escovopsis isolates are better suited to consume L. gongylophorus aggressively and efficiently in pure culture, such as AP090209-01, AP090731-01, RM090730-02, AP090225-01, DE090731-01 and AP100526-01. However, other strains evaluated, such as DE100121-01, RV100727-06, RV100726-14, RM100121-04, AP100718-01, RV100727-03, DE100121-02, RM100121-02 and DE100719-02, showed a significantly lower growth rate in this assay. It is possible that these strains will also require longer times to establish a successful infection in field colonies, thus allowing the ants to remove them from the fungus garden (Currie & Stuart, 2001). This reduced virulence does not seem to be caused by the cultivar defensive compounds, as we did not detect any statistically significant evidence for a successful defence strategy employed by L. gongylophorus. As we used cultivars from Atta species exclusively, these results are congruent with previous research showing that L. gongylophorus is better suited to defend itself against Escovopsis strains isolated from different attine ant genera (Gerardo et al., 2006a) and further confirm that the parasite is well adapted to avoid its particular host defence mechanisms (Currie, 2001b).

The results discussed above are also confirmed by the fungus garden piece bioassay, in which we evaluated virulence against L. gongylophorus employing an aggressiveness scale (Table 1) in a more complex setting that includes the plant material processed by the ants, other symbiotic micro-organisms (Pinto-Tomás et al., 2009; Suen et al., 2010) and two minor ant workers. We found significant differences in the ability of the tested Escovopsis strains to damage the inoculated fungus garden piece. Further, the most virulent isolates in the pure culture bioassay were also the most aggressive when consuming the fungus garden piece, whilst the origin of the fungus garden piece did not have any significant influence on the results (Fig. 1). However, ~50% of Escovopsis isolates did not grow at all on the inoculated fungus garden; this could be due to the

Table 2. Summary of EF-1α sequencing of Escovopsis isolated from four locations in Costa Rica

<table>
<thead>
<tr>
<th>Region</th>
<th>No. samples sequenced</th>
<th>No. base pairs</th>
<th>Mean genetic distance in each region (D)</th>
<th>Mean genetic distance among all sequences (D)</th>
</tr>
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<tr>
<td>Turrialba</td>
<td>2</td>
<td>858</td>
<td>0</td>
<td>0.0157</td>
</tr>
<tr>
<td>Upala</td>
<td>11</td>
<td>858</td>
<td>0.0167</td>
<td>0.0157</td>
</tr>
<tr>
<td>Sarapiqui*</td>
<td>11*</td>
<td>858</td>
<td>0</td>
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</tr>
<tr>
<td>Osa</td>
<td>2</td>
<td>858</td>
<td>0</td>
<td>0.0157</td>
</tr>
</tbody>
</table>

*Two Escovopsis sequences from Trachymyrmex and Apterotigsta colonies (Sarapiqui) were not included.
ants’ defensive behaviour. Previous research has shown that as soon as Escovopsis is detected, the ants remove contaminated portions of the fungus garden by detaching, ingesting and neutralizing them in their infrabuccal pockets, before discarding them in a safer location (Currie & Stuart, 2001). Therefore, if the parasite’s spores do not germinate rapidly enough, the ants will remove them and suppress their growth, whilst the most successful isolates’ spores will germinate early to start a successful infection process (see detailed daily data in Table S5). Taken together, our results indicate that there are certain Escovopsis strains better suited to avoid the defence mechanisms evaluated so far. Therefore, the 10 most virulent strains were selected to evaluate their antagonistic abilities in a more complex bioassay.

The subcolony bioassay is a more realistic representation of the interactions that occur in nature, as it involves both medium and minor ants (major workers usually do not engage in sanitation of the fungus garden), a healthy and intact fungus garden that includes ant brood and associated micro-organisms, and the availability of fresh leaf material to cultivate the fungus. Therefore, some Escovopsis strains that showed a high antagonistic activity in previous bioassays did not accomplish the same potential after confronting a subcolony, as manifested by AP909209-01 and RM907030-01 (Fig. 2). However, the collective defence imposed by medium and minor workers, the antibiotic-producing bacteria and the cultivar itself were incapable of suppressing most of the Escovopsis strains, and some of these parasites grew significantly more rapidly than others.

In combination, the three bioassays employed allowed us to select the three most virulent Escovopsis strains for further research into their potential as biocontrol agents. Interestingly, all of them cluster together in a well-defined clade in our phylogenetic analysis (Fig. 3a). On the contrary, the origin of the diverse Escovopsis strains had no influence in the clustering pattern observed in the constructed phylogeny. Previous studies also showed no correlation between Escovopsis phylogeny and geographical distribution, indicating lineage mixing across large geographical areas over extensive time periods (Currie et al., 2003). At the scale applied here, the lack of a geographical pattern suggests that local genotypes are not adapted to a particular site, which could be due to a lack of genetic variation or to the parasite migrating too far and too fast to specialize on local populations (Lively, 1999). As expected for a successful parasite, here we show that there is enough genetic and phenotypic variation in Escovopsis populations to identify and select virulent Escovopsis genotypes, and these genotypes are likely to be highly toxic for most colonies across different geographical locations.

As opposed to Escovopsis, there is little genetic variation in L. gongylophorus cultured by Atta and Acromyrmex, which basically grow the same fungal clone which relies exclusively on vertical transmission by queens (Chapela et al., 1994; Mueller et al., 1998), unlike basal attines, whose agriculture comprises a diverse group of basidiomycetes (Schultz & Brady, 2008). Therefore, it is not surprising that all isolates belonged to the same clade in our phylogenetic analysis (Fig. 3b) and that they did not have a significant effect on the outcome of the bioassays despite their different origins across Costa Rica. Growing a clonal monoculture to feed millions of insects in a colony allows the ants to focus all their energy on solving the needs of their specific clone, avoiding resource competition among different lineages, but it also makes their valuable crop more susceptible to pathogens and parasites (Currie et al., 1999a). Identifying the most virulent strains of those parasites – a feasible task for the specialized and coevolved Escovopsis – is the first step towards designing a sustainable and effective integrated pest management strategy based on biological control strategies, in order to reduce the negative economic and environmental impact of leaf-cutter ants in Latin American agriculture.

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