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Variation in *Pseudonocardia* antibiotic defence helps govern parasite-induced morbidity in *Acromyrmex* leaf-cutting ants

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Summary

Host–parasite associations are potentially shaped by evolutionary reciprocal selection dynamics, in which parasites evolve to overcome host defences and hosts are selected to counteract these through the evolution of new defences. This is expected to result in variation in parasite-defence interactions, and the evolution of resistant parasites causing increased virulence. Fungus-growing ants maintain antibiotic-producing *Pseudonocardia* (*Actinobacteria*) that aid in protection against specialized parasites of the ants' fungal gardens, and current evidence indicates that both symbionts have been associated with the ants for millions of years. Here we examine the extent of variation in the defensive capabilities of the ant–actinobacterial association against *Escovopsis* (parasite-defence interactions), and evaluate how variation impacts colonies of fungus-growing ants. We focus on five species of *Acromyrmex* leaf-cutting ants, crossing 12 strains of *Pseudonocardia* with 12 strains of *Escovopsis* in a Petri plate bioassay experiment, and subsequently conduct subcolony infection experiments using resistant and non-resistant parasite strains. Diversity in parasite-defence interactions, including pairings where the parasites are resistant, suggests that chemical variation in the antibiotics produced by different actinobacterial strains are responsible for the observed variation in parasite susceptibility. By evaluating the role this variation plays during infection, we show that infection of ant subcolonies with resistant

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Fig. S1. Cultivar–*Escovopsis* Petri plate bioassay. The average number of days the 12 *Acromyrmex*-associated *Escovopsis* isolates took to overgrow cultivars and sporulate when presented with cultivars from the same 12 colonies in an *in vitro* Petri plate bioassay. Different colours indicate the average number of days: white: 14+ days, light yellow: 12–13 days, yellow: 10–11 days, orange: 8–9 days, and red: 6–7 days ($n = 3$).

Fig. S2. Relationships between *Pseudonocardia* size and *Escovopsis* degree of inhibition. The association between *Pseudonocardia* size and the zone of inhibition (ZOI) in the *Pseudonocardia*–*Escovopsis* pairings ($r = 0.12$; $R^2 = 0.0152$).

Table S1. Results of the statistical evaluation of *Escovopsis*–cultivar bioassay. The top section of the table gives the nested model, having cultivar and *Escovopsis* clones nested within their respective ant species origins. The bottom section of the table gives the analyses excluding ant origins, and evaluating only the symbiont clones individually and their interaction. Both analyses were performed under a gamma distribution and significance values evaluated with likelihood ratio statistics for a Type III analysis.

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parasite strains results in significantly higher parasite-induced morbidity with respect to garden biomass loss. Our findings thus further establish the role of *Pseudonocardia*-derived antibiotics in helping defend the ants' fungus garden from the parasite *Escovopsis*, and provide evidence that small molecules can play important roles as antibiotics in a natural system.

Introduction

The biology of all living organisms, including humans and human-domesticated plants and animals, is influenced by parasites (Jaenike, 1978; Price *et al.*, 1986; Ewald, 1994; Cohen, 2000; Palumbi, 2001). The constant selective pressures imposed by parasites on hosts have made them a major force influencing evolution and species diversification (Jaenike, 1978; Ewald, 1994; Cohen, 2000; Palumbi, 2001). On an evolutionary time scale, natural host–parasite associations are shaped, at least in part, by arms race-like dynamics: parasites evolve to overcome host defences and hosts are selected to counteract these through the evolution of new defences (Van Valen, 1973; Hamilton, 1980). In humans, the dynamics of host–parasite interactions are modified through the application of bacteria-derived (Clardy *et al.*, 2005) antimicrobial chemicals. However, the efficacy of these compounds in human medicine and agriculture is continuously threatened by the emergence of resistant parasite phenotypes, which arise as a consequence of selective pressures imposed by the applied antimicrobials (Cohen, 2000; Palumbi, 2001; Davies, 2007). Recent work has revealed that some insects also employ bacteria-derived antibiotics for protection against parasites (fungus-growing ants: Currie *et al.*, 1999a; Oh *et al.*, 2009; European beewolves: Kaltenpoth *et al.*, 2005; and Southern Pine beetles: Scott *et al.*, 2008). Theory predicts that the target parasites of the bacteria-derived antibiotic compounds should evolve resistance, but the presence and impact of such resistant parasites have not been evaluated in any of these natural systems. Here, we determine the potential for the evolution of parasite resistance by determining whether: (i) there is variation in parasite resistance, and (ii) this variation leads to differential parasite success. Fungus-growing ants in the tribe Attini (Hymenoptera: Formicidae) engage in an obligate mutualism with fungi they cultivate for food (Agaricales: Lepiotaceae and Pterulaceae) (Chapela *et al.*, 1994; Munkacsí *et al.*, 2004; Schultz and Brady, 2008). This beneficial ant–fungus symbiosis is parasitized by microfungi in the genus *Escovopsis* that attack and consume the ants' fungal garden (Hypocreales: Ascomycota) (Currie *et al.*, 1999a; 2003b; Reynolds and Currie, 2004). The cultivated fungi can suppress the growth of *Escovopsis* strains not known to infect them in nature, but cannot inhibit isolates of naturally occurring parasites (Gerardo *et al.*, 2006a,b; Gerardo and Caldera, 2007; Supporting information; Fig. S1). Instead, to defend their fungus gardens from *Escovopsis*, the ants rely on behavioural removal of *Escovopsis* spores and mycelium (Currie and Stuart, 2001), secretions from worker's metapleural glands (Bot *et al.*, 2002; Fernández-Marín *et al.*, 2009), and mutualistic antibiotic-producing filamentous bacteria in the genus *Pseudonocardia* (Actinomycetales: Pseudonocardiaceae; Currie *et al.*, 1999b; Cafaro and Currie, 2005) (Fig. 1A). The relative contribution of these different defence mechanisms likely depends on the genus of fungus-growing ant. In the ant genus *Acromyrmex*, artificially removing *Pseudonocardia* from the ant cuticle results in significantly larger negative effects of *Escovopsis*, indicating that *Pseudonocardia* is an important defence against these parasites (Currie *et al.*, 2003a). *Actinobacteria* in other genera have also been isolated from ant fungus gardens (Mueller *et al.*, 2008; Haeder *et al.*, 2009), and in culture some strains have been shown to produce small molecules that inhibit the growth of *Escovopsis* (Haeder *et al.*, 2009). However, it remains to be established if these bacteria are more than transient community members (i.e. being isolated because they are present as non-metabolizing inocula introduced to the garden with the leaf material added by workers), or instead are capable of growth and production of these small molecules within the garden matrix (see *Results and discussion*). Individual *Acromyrmex* ant colonies

appear to associate with a single *Pseudonocardia* strain (Poulsen *et al.*, 2005), which is persistently present within nests (at least in laboratory colonies) for extended periods of time (years) (••. Marsh, M. Poulsen and C.R. Currie, unpubl. data), but there is genetic diversity in this bacterial symbiont both within and between ant species (Cafaro and Currie, 2005; Poulsen *et al.*, 2005; Mikheyev *et al.*, 2008). The between-colony diversity in ant-associated *Pseudonocardia* leads to the prediction that different *Pseudonocardia* strains have different inhibitory capabilities against different *Escovopsis* strains (Currie *et al.*, 2006).

In order to build on our knowledge of the role of *Pseudonocardia* in the association with fungus-growing ants, we examine how differences in inhibitory capabilities between different *Pseudonocardia* strains affect *Escovopsis*-induced morbidity. We do this by linking observed *Pseudonocardia*–*Escovopsis* interactions *in vitro* to *in vivo* defensive capabilities during subcolony infection. Capitalizing on the ability to culture the microbial symbionts associated with *Acromyrmex* leaf-cutting ants, we examine interactions between the antibiotic-producing bacteria and the target parasites in a bioassay experiment, crossing strains associated with 12 colonies from five species of *Acromyrmex* leaf-cutting ants (Fig. 1A and B). Based on the variation in *Escovopsis*–*Pseudonocardia* (parasite–defence) interactions *in vitro*, we test the prediction that the inhibitory capabilities of *Pseudonocardia*-derived antibiotics correlate with parasite-induced morbidity in fungus-growing ant nests *in vivo*. We do this by performing an infection experiment pairing colonies with *Escovopsis* strains susceptible or resistant to the antibiotics produced by the resident bacterium.

Results and discussion

Our symbiont pairings, evaluating the variation in parasite–defence interactions in a Petri plate bioassay experiment of *Pseudonocardia* and *Escovopsis* from 12 colonies distributed across five *Acromyrmex* species, revealed abundant variation in suppression of *Escovopsis* (Fig. 1B). The degree of *Escovopsis* inhibition observed depended on the specific combination of strains of *Pseudonocardia* and *Escovopsis* paired. The reason for this variation is likely a combination of (i) the susceptibility of *Escovopsis*, (ii) compound differences between *Pseudonocardia* strains, (iii) compound production rate differences between *Pseudonocardia* strains, and (iv) potential differences between bacterial strains in their composition of compounds when more than a single compound is produced. This is supported by our findings of a statistically strong effect of the interaction between *Pseudonocardia* inhibitory capabilities and the susceptibility of *Escovopsis* (likelihood ratio statistics for a Type III two-way ANOVA: $\chi^2 = 202.5$, d.f. = 104, $P < 0.0001$) (Table 1). The analysis further identified significant strain effects of both *Escovopsis* (likelihood ratio statistics for a Type III nested ANOVA: $\chi^2 = 14.50$, d.f. = 7, $P = 0.0429$) and *Pseudonocardia* ($\chi^2 = 114.9$, d.f. = 7, $P < 0.0001$) (Table 1). The variation observed included pairings in which the parasite *Escovopsis* exhibited significant resistance to the *Pseudonocardia*-derived antibiotics. *Escovopsis* growth was completely suppressed by the antibiotic-producing *Actinobacteria* in 54.2% of bioassay pairings, but *Escovopsis* was not suppressed, indicating antibiotic resistance, in 11.8% of the bioassay pairings (Fig. 1B). Despite abundant variation in interactions within and between ant species, there were notable effects of the ant species origin of both symbionts (*Pseudonocardia* $\chi^2 = 41.41$, d.f. = 4, $P < 0.0001$; *Escovopsis*: $\chi^2 = 21.21$, d.f. = 4, $P = 0.0003$; Table 1). Our analysis also indicated a possible dose effect ($\chi^2 = 9.59$, d.f. = 1, $P = 0.002$), but this correlation explained only 1.5% of the variation ($r = 0.12$; Fig. S2). The presence of resistance in the parasite and variation in bacteria-derived chemistry suggests that interactions on this finer phylogenetic scale shape host–parasite dynamics within this system. In fact, 45.3% (29 out of 64) of pairings between symbionts isolated from the two sympatric *Acromyrmex* species (*A. echinator* and *A. octospinosus*) showed weak or no inhibition [zone of inhibition (ZOI)

< 1.0 cm] in the bioassay, indicating that resistant *Escovopsis* phenotypes do infect leaf-cutting ant nests in nature.

In order to examine whether the patterns of inhibition of *Escovopsis* observed reflect general antifungal properties of the *Pseudonocardia* strains tested, we performed a supporting experiment testing the antifungal effects of the 12 strains against a strain of *Candida albicans* (strain 1002, see Supporting information for more details). We used the same experimental Petri plate bioassay set-up as in the *Pseudonocardia*–*Escovopsis* pairings, except that *C. albicans* was inoculated in a liquid suspension, and found that none of the 12 *Pseudonocardia* strains displayed visible inhibition of *C. albicans*. Although we cannot rule out that inhibition of *C. albicans* could occur at higher concentrations of *Pseudonocardia*-derived secondary metabolites, this set-up allows for a direct comparison with the *Pseudonocardia*–*Escovopsis* assay. This suggests that the variation in *Escovopsis* inhibition between *Pseudonocardia* strains is not a result of differences in the levels of general antifungal activity between different bacterial strains (for details, see Supporting information).

Based on the Petri plate bioassay results, we predicted that the extent of parasite-induced garden biomass loss would depend upon the combination of *Pseudonocardia* and *Escovopsis* parasite strain. To test this prediction, we performed a subcolony experiment pairing six different ant colonies with three different *Escovopsis* strains, choosing pairings that included the range of antagonistic displays of *Pseudonocardia* symbionts towards *Escovopsis* *in vitro* (Fig. 1B). *Escovopsis* impact was evaluated as fungus garden mass loss 1 week after *Escovopsis* application. *Escovopsis*-induced garden biomass loss indeed depended on the combination of the resident *Pseudonocardia* associated with the host ant colony and the strain of *Escovopsis* (Fig. 1C). The *in vivo* evaluation revealed that *Escovopsis* rapidly overgrew all cultivar fragments in the absence of ants (one-way ANOVA: $F_{7,752} = 11\,240$, $P < 0.0001$; Table 2). In the presence of ants, the impact of *Escovopsis* was strongly correlated with the inhibition, or lack thereof, displayed in the *in vitro* bioassay by the resident *Pseudonocardia* bacterium, indicating that reactions *in vitro* represent the effectiveness of bacterial defence *in vivo* ($F_{2,123} = 9.66$, $P < 0.0001$) (Fig. 1C; Table 2). Of particular interest were situations where *Escovopsis* strains were only weakly inhibited by the bacterium *in vitro* (ZOI < 1.0 cm; 25.7% of the pairings), as these combinations, on average, showed virulence levels *in vivo* that were comparable to pairings with strongly resistant *Escovopsis* strains (Fig. 1C) ($F_{1,83} = 0.2819$, $P = 0.5969$). A two-way ANOVA confirmed the isolated effects of ant colony ($F_{5,135} = 5.74$, $P < 0.0001$) and *Escovopsis* treatment ($F_{6,135} = 23.53$, $P < 0.0001$), and further showed a marginally non-significant interaction between the two ($F_{15,135} = 1.64$, $P = 0.071$) (Table 2). The results of this experiment indicate that the inhibitory capabilities of the antimicrobials produced by the resident *Pseudonocardia* bacterium directly influence the impact of *Escovopsis* during colony infections. Thus, strains of the garden parasite exhibiting *in vitro* resistance to *Pseudonocardia* secondary metabolites cause greater morbidity to the ants' fungus garden *in vivo*, confirming that the symbiont bioassay pairings reflect interactions within colonies.

Further, our infection experiment indicates that even strains of *Escovopsis* exhibiting some, but not strong, resistance *in vitro* (i.e. growth suppression < 1.0 cm ZOI) cause higher levels of fungus garden morbidity *in vivo*. This indicates that more than a third of the parasite-defence pairings screened in the bioassay exhibit a degree of resistance to the antibiotics produced by the bacteria that would result in increased garden morbidity. Importantly, resistance was present in *Escovopsis*–*Pseudonocardia* combinations found in nature, and even occurred, although only rarely, in pairings between symbionts from the same individual ant colony (Fig. 1B). While it may appear counterintuitive to find surviving colonies inhabited by resistant parasite strains, other defences in the ants (e.g. grooming and

weeding; Currie and Stuart, 2001) likely compensate for this lack of efficient *Actinobacteria*-defence and thus help prevent complete colony collapse in such rare cases. Despite the presence of resistant parasite strains, a large portion of pairings in the bioassay experiments exhibited strong suppression of the parasite (62.5%) (Fig. 1B), and infection with parasite strains susceptible to the *Actinobacteria* exhibited less garden biomass loss (Fig. 1C). This implies that the costs incurred by the ants to maintain *Pseudonocardia*, through specialized exocrine glands that secrete the substrate for *Pseudonocardia* growth (Poulsen *et al.*, 2003; Currie *et al.*, 2006), are outweighed by the benefits obtained by the ability of *Pseudonocardia* to suppress *Escovopsis* during colony infections.

Naturally occurring resistant parasite strains, like those identified in this study, should impose a selective pressure on the ant host for the evolution of novel defences. The ant–*Pseudonocardia* association appears to maintain efficient defence against *Escovopsis* despite the evolution of antibiotic resistance. The mechanisms behind the successful application of *Actinobacteria*-derived antibiotics by fungus-growing ants are unclear, but several scenarios can be envisioned. The first conceivable scenario is that parasite-defence dynamics in the fungus-growing ant symbiosis are shaped by antagonistic co-evolution, involving the evolution of resistance in the parasite, counteracted by the evolution of novel compounds by *Pseudonocardia*. Specifically, this posits that the selective pressure imposed by *Escovopsis* on *Pseudonocardia* results in the *Pseudonocardia*-derived antibiotics evolving in parallel with the target parasites. In turn, the antibiotic secretions by *Pseudonocardia* targeted at *Escovopsis* strains are expected to impose a selective pressure on *Escovopsis* to evolve resistance (Cohen, 2000; Palumbi, 2001; Davies, 2007). The presence of such antagonistic co-evolution would result in variability in the chemistry of *Pseudonocardia* secretions, as our bioassay pairings suggest (Fig. 1B). The relatively short generation time of bacteria may allow for a fast rate of change, but our understanding of changes in the metabolic capacities of the bacterium residing in individual nests is, as of yet, limited. Such change either may be restricted to include only minor modifications of existing secondary metabolites, and not major and elaborate alterations, or may involve the possibility of acquiring new antibiotic-coding gene clusters through horizontal gene transfers (Ginolhac *et al.*, 2005; Jenke-Kodama *et al.*, 2005; Schmitt and Lumbsch, 2009). An alternative mechanism for rapid modifications is the acquisition of a novel *Pseudonocardia* strains, with novel secondary metabolites, by an ant colony. Co-phylogenetic patterns between *Pseudonocardia* and fungus-growing ants suggest relatively frequent switching of the *Pseudonocardia* symbiont between species, and even genera (Poulsen *et al.*, 2005; Mikheyev *et al.*, 2008), as well as several acquisitions of *Pseudonocardia* from free-living (non-fungus-growing ant-associated) counterparts over the evolutionary history of the association (Cafaro and Currie, 2005). Both evolution of novel *Pseudonocardia* antibiotics and acquisition of novel antibiotic-producing bacteria from the environment likely play a role in mediating host–pathogen dynamics in this system.

It is generally recognized that just because one microbe inhibits another in Petri plate challenges, this may imply, but does not prove, that the compound produced causes inhibition in nature (Waksman, 1961; Davies, 2006; Clardy *et al.*, 2009). In fact, it has been argued that what we consider antibiotics do not function as such in nature, but instead are signalling compounds (Davies, 2006; Yim *et al.*, 2007). Recently, it has been shown that *Actinobacteria* genera other than *Pseudonocardia* (mostly *Streptomyces*) have the potential to inhibit *Escovopsis* (Mueller *et al.*, 2008; Haeder *et al.*, 2009). However, these studies are only supported by bioassays, not infection experiments, and thus implies, but does not show, that these *Actinobacteria* could play a similar role in the fungus-growing ant symbiosis. In contrast, the role of *Pseudonocardia*-derived antibiotics for dealing with *Escovopsis* infections is now supported through both removal and infection experiments (Currie *et al.*, 2003a) and through the evaluation of the role inhibitory capabilities play in governing parasite-induced morbidity (this study). For the two main species of *Acromyrmex* focused

on in this study, *A. octospinosus* and *A. echinator*, it is well established that *Pseudonocardia* is vertically transmitted between generation by incipient queens (Currie *et al.*, 1999a), and that specific *Pseudonocardia* strains are associated with these ant species (Cafaro and Currie, 2005; Poulsen *et al.*, 2005). In addition, the same *Pseudonocardia* strain can be isolated from the exoskeleton of workers in individual *Acromyrmex* colonies maintained in the lab for several years, indicating consistent and persistent associations with *Pseudonocardia* (••. Marsh, M. Poulsen and C.R. Currie, unpubl. data). Haeder and colleagues (2009) also isolated *Pseudonocardia* as the main bacterium from workers of *A. octospinosus* and *A. echinator*; they only found *Streptomyces* in the fungus garden matrix. Although these *Streptomyces* strains potentially inhibit *Escovopsis* in Petri plates, it remains to be determined if they represent more than transient microbes that do not actively metabolize within the ant symbiosis (see Caldera *et al.*, 2009).

Given the metabolic capacity of *Actinobacteria* to produce antibiotic (e.g. Demain, 1999), in addition to the relatively short generation time of bacteria allowing for faster rates of change, it is perhaps not surprising that the defensive mutualist in the association is an *Actinobacterium*. This suggests that the ant–*Pseudonocardia* mutualism is an example of symbiosis as evolutionary innovation: the ants benefit from the mutualism by gaining access to a source of rapidly changing metabolites with potent antimicrobial properties. The development of novel antibiotics by *Pseudonocardia* strains may evolve in response to *Escovopsis* parasites, thereby counteracting the evolution of parasite resistance. The availability of potent antibiotic producers to modify their secondary metabolism and/or the ants' ability to acquire new sources of antibiotics from other ant-associated or free-living bacteria may have allowed for a more efficient defence against *Escovopsis* than would have been achieved by the ants alone. Long-term antibiotic use in ants may only have remained stable and efficient over time due to their ability to access microbes from the environment that harbour diverse antimicrobial properties. Whether or not novel chemistries are obtained from the resident ant-associated bacterium, or via acquisition forming novel associations, our findings illustrate the benefits of this type of defensive mutualism in the ability to counteract resistance evolution in the target parasite.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A. Leaf-cutting ant worker with *Pseudonocardia*-covered cuticle. Micrograph of an *Acromyrmex octospinosus* major worker illustrating the abundance of mutualistic bacteria on the ant cuticle. B. *Acromyrmex*-associated *Pseudonocardia*–*Escovopsis* bioassay. Results of a symbiont pairing bioassays between 12 strains of *Pseudonocardia* (vertical) and *Escovopsis* (horizontal) associated with five species of *Acromyrmex* leaf-cutting ants. These included three Argentinean species: *A. niger* (colony code CC030327-02), *A. hispidus fallax* (SP030327-01 and UGM030327-02) and *A. laticeps* (UGM030330-04), and two Panamanian species: *A. echinator* (Ae291, Ae292, Ae295 and CC031212-01) and *A. octospinosus* (CC011010-04, CC031210-22, ST040116-01, UGM020518-05). Pure culture *Pseudonocardia* were point inoculated in the centre of plates containing PDA without antibiotics and were grown for 3 weeks until reaching a diameter of c. 1–1.5 cm, after which *Escovopsis* was inoculated at the edge of the plate. Plates were examined bi-weekly and when a clear zone of inhibition (ZOI) had formed in a given pairing (typically within 2–3 weeks after *Escovopsis* inoculation), the ZOI between symbionts (Cafaro and Currie, 2005) was recorded. Each box represents the average ZOI ($n = 3$) of a given pairing and different colours indicate the degree of inhibition: white: ZOI = 0 cm, light yellow: ZOI = 0.01–0.50 cm, yellow: ZOI = 0.51–1.0 cm, orange: ZOI = 1.01–1.5 cm, and red: ZOI > 1.51 cm. Bioassay pairings enclosed in boxes represent the pairings from which subcolonies were created and inoculated with *Escovopsis* (Fig. 1C). C. Subcolony evaluation of *Escovopsis* virulence. The *in vivo* virulence of *Escovopsis* measured as fungus garden biomass loss when miniature colonies, created using a standard set-up (Bot *et al.*, 2001; Poulsen and Boomsma, 2005) with 80 mg of fungus garden and two major *Acromyrmex* workers covered with the *Pseudonocardia* bacterium (abundance scores 9–12 in Poulsen *et al.*, 2003), were exposed to *Escovopsis* infection. Subcolonies were housed in 60 ml plastic cups with small holes in the lids to provide air, moist tissue in the bottom to assure high humidity, and a folded oak leaf to provide forage for the ants. To assure that *Escovopsis* spore inoculations did not differ between subcolonies, we quantified spore concentrations in aqueous

suspensions containing filter-sterilized water and 0.05 µl Tween 20 per ml water prior to application, and subsequently inoculated individual subcolonies with an amount of suspension corresponding to $11\,500 \pm 252$ spores (mean $\pm$ SE; $n = 17$). Negative control subcolonies were applied the same volume of filter-sterilized water and Tween 20 without *Escovopsis* spores. Fungus garden weight was measured 1 week after *Escovopsis* application. Fungus garden loss is averaged across three strength levels of *in vitro* *Escovopsis* inhibition by *Pseudonocardia* (strong: ZOI > 1.0 cm, $n = 48$; weak: ZOI = 0.01–1.0 cm, $n = 42$; or no: ZOI = 0 cm, $n = 36$; Fig. 1B).

Table 1

Results of the statistical evaluation of the within-*Acromyrmex Pseudonocardia-Escovopsis* bioassay.

	χ^2	d.f.	P
Nested model			
<i>Pseudonocardia</i> strain (ant species)	114.9	7	< 0.0001
<i>Escovopsis</i> strain (ant species)	14.50	7	0.0429
Ant species of <i>Pseudonocardia</i>	41.41	4	< 0.0001
Ant species of <i>Escovopsis</i>	21.21	4	0.0003
Ant species of <i>Pseudonocardia</i> * ant species of <i>Pseudonocardia</i>	28.14	15	0.0207
Size of <i>Pseudonocardia</i>	9.590	1	0.002
Two-way ANOVA			
<i>Pseudonocardia</i> strain	233.8	11	< 0.0001
<i>Escovopsis</i> strain	77.83	11	< 0.0001
<i>Pseudonocardia</i> strain * <i>Escovopsis</i> strain	202.5	104	< 0.0001
Size of <i>Pseudonocardia</i>	1.930	1	0.1616

Due to the non-normality of data and the high sensitivity of imputing, zero-values in log-linear models, we employed a generalized linear model in which zero-values were replaced with the value, 0.00001 and significance of terms in the models was determined through likelihood ratio statistics for Type III ANOVAs. The analysis was performed in SAS v. 9.1.3 (SAS Institute, 1994). Two models were built: the first model contained the respective ant species from which *Pseudonocardia* and *Escovopsis* strains originated from, *Pseudonocardia* and *Escovopsis* strains nested within their ant species origins, the interaction term between the ant species symbionts originated from, and the size of *Pseudonocardia* at the time inhibition of *Escovopsis* was scored (top section). Since this nested design did not allow for an evaluation of the interaction between *Pseudonocardia* and *Escovopsis* strains, we performed a separate analysis including only *Pseudonocardia* and *Escovopsis* strains and their interaction (bottom section).

Table 2Statistical analyses of subcolony evaluation of *Escovopsis* virulence in *Acromyrmex*.

	d.f.	F	P
Two-way ANOVA			
Colony origin	5,135	5.740	< 0.0001
<i>Escovopsis</i> strain	6,135	23.53	< 0.0001
Colony origin * <i>Escovopsis</i> strain	15,135	1.640	0.071
One-way ANOVAs			
Absence of ants	11,240	7.752	< 0.0001
No, weak or strong actinobacterial inhibition <i>in vitro</i>	2,123	9.660	< 0.0001
No or weak actinobacterial inhibition <i>in vitro</i>	1,83	0.2819	0.5969
<i>Escovopsis</i> strain	5,120	2.532	0.0323
Ant colony	5,120	6.331	< 0.0001

The top section of the table shows the results of a two-way ANOVA evaluating the overall effects of *Escovopsis* on subcolonies of *Acromyrmex* ants *in vivo*. The bottom section represents four one-way ANOVAs testing the isolated effects of (i) ant presence in subcolonies, (ii) whether the *Pseudonocardia* symbiont associated with the ant colony exhibited strong (ZOI > 1.0 cm), weak (ZOI = 0–0.9 cm) or no (ZOI = 0 cm) *in vitro* inhibition of the *Escovopsis* strain, (iii) whether there was a difference in *Escovopsis* virulence between subcolonies harbouring *Pseudonocardia* strains exhibiting weak (0 < ZOI < 0.9 cm) or no (ZOI = 0) *in vitro* inhibition of the *Escovopsis* strain, (iv) individual *Escovopsis* strains, and (v) ant colony.