Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria

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Fungus-growing ants (tribe Attini) engage in a mutualism with a fungus that serves as the ants’ primary food source, but successful fungus cultivation is threatened by microfungal parasites (*genus Escovopsis*). Actinobacteria (*genus *Pseudonocardia*) associate with most of the phylogenetic diversity of fungus-growing ants; are typically maintained on the cuticle of workers; and infection experiments, bioassay challenges and chemical analyses support a role of *Pseudonocardia* in defence against *Escovopsis* through antibiotic production. Here we generate a two-gene phylogeny for *Pseudonocardia* associated with 124 fungus-growing ant colonies, evaluate patterns of ant–*Pseudonocardia* specificity and test *Pseudonocardia* antibiotic activity towards *Escovopsis*. We show that *Pseudonocardia* associated with fungus-growing ants are not monophyletic: the ants have acquired free-living strains over the evolutionary history of the association. Nevertheless, our analysis reveals a significant pattern of specificity between clades of *Pseudonocardia* and groups of related fungus-growing ants. Furthermore, antibiotic assays suggest that despite *Escovopsis* being generally susceptible to inhibition by diverse Actinobacteria, the ant-derived *Pseudonocardia* inhibit *Escovopsis* more strongly than they inhibit other fungi, and are better at inhibiting this pathogen than most environmental *Pseudonocardia* strains tested. Our findings support a model that many fungus-growing ants maintain specialized *Pseudonocardia* symbionts that help with garden defence.

**Keywords:** fungus-growing ants; microbial interaction; mutualism; *Pseudonocardia*; symbiosis

1. INTRODUCTION

Symbiosis, an intimate association between different species, is a major theme in biology. This is exemplified by many of the major transitions in the history of life that appear to have occurred through the formation of beneficial symbioses (*e.g.* the origin of eukaryotes and the colonization of land by plants) \([1,2]\). Host–microbe symbioses express different degrees of specificity and specialization that can result in restricted association of one or both partners. Some show low partner fidelity (*e.g.* arbuscular mycorrhizal fungi), while other hosts associate with specific symbiont clades or species (*e.g.* boxtail squid–*Vibrio fischeri*; legumes–rhizobia; entomopathogenic nematodes–bacteria). For associations that are not strictly vertically transmitted, patterns of specificity between hosts and symbionts can arise via physiological or ecological mechanisms \([3–5]\). These species-specificity factors likely evolved through reciprocal selection (*i.e.* coevolution) and are predicted to result in codiversification between partners \([6,7]\). However, symbiont switches between related hosts are typically frequent enough to prevent cospeciation, without disrupting functional integration between partners, and potentially result in patterns of diffuse coevolution in which groups of related hosts associate with groups of related symbionts (codiversification) \([8]\).

The ancient and obligate mutualism between fungus-growing ants (tribe Attini) and their fungal cultivars is
an example of a symbiosis shaped by millions of years of diffuse coevolution [9]. The ants cultivate fungi for food (Basidiomycota; Agaricales: Lepiotaceae and Pterulaceae) [10] in specialized gardens. In exchange for the fungus serving as the ants’ primary food source, the ant host provides the fungus with protection, substrate for growth and dispersal to new nests through vertical transmission [11]. Since its origin, approximately 50 million years ago, the tribe Attini has diversified into more than 230 described species in 12 genera [11]. Specific groups of ants specialize on cultivating specific groups of fungi, a pattern reflecting a significant degree of codiversification within the mutualism [9–11]. These combinations of specific attine ants and their fungi have been classified into five discernible agricultural systems [11]. The most basal groups of ants in the Palaeoattini and basal Neoattini cultivate fungi from the Lepiotaceae family, which is considered ‘Lower agriculture’. A few ant species in the Palaeoattini genus *Apterostigma* secondarily switched to cultivating fungi in the family Pterulaceae (‘Coral fungus agriculture’). In the Neoattine genus *Cyphomyrmex*, some species cultivate a yeast form of Lepiotaceous fungi (‘Yeast agriculture’). The ‘Higher agriculture’ system includes the most recent transition in ant fungiculture, the origin of ‘Leaf-cutter agriculture’ by ants in the genera *Atta* and *Acromyrmex*, which exclusively use fresh plant material to cultivate their fungus (see [11]; figure 1).

A third symbiont, microfungi in the genus *Escovopsis* (Ascomycota; Hypocreales), also shares a pattern of specialization within the fungus-growing ant–microbe association [12,13]. *Escovopsis* species exploit the fungal cultivar as their nutrient source, they are horizontally transmitted between colonies, and they can form persistent and virulent infections [12]. The garden parasite appears to have had a single and ancient origin in the ant–fungus mutualism, with subsequent codiversification with the ants and their cultivar, so that specific groups of ant–cultivar pairings are infected by specialized *Escovopsis* clades [13–15]. On a broad phylogenetic scale, at least one evolutionary switch across agriculture systems appears to have occurred, and, at a finer phylogenetic scale, closely related strains of *Escovopsis* switch between cultivars of closely related ant species and genera [15,16].

To defend their fungus gardens from infections, attine ants have evolved specialized defences, including fungus grooming, weeding, metapleural gland use and general nest hygiene [17–19]. In addition, the ants engage in a symbiotic association with Actinobacteria in the genus *Pseudonocardia*, which produce antibiotics that inhibit the growth of *Escovopsis* [20–22]. Evidence supporting the role of *Pseudonocardia* in helping protect ant fungus gardens includes: (i) bioassays demonstrating bacterial inhibition of *Escovopsis* in vitro across most of the phylogenetic diversity of the association ([20,22,23], this study); (ii) structural identification of a novel chemical compound responsible for inhibition of *Escovopsis* by an *Apterostigma*-associated *Pseudonocardia* symbiont [24]; (iii) two separate in vitro infection experiments in the fungus-growing ant genus *Acromyrmex* [21,22], and (iv) the finding that experimental infections of *Apterostigma* colonies with a black yeast parasite of the ant bacterium association reduces the ability of *Pseudonocardia* to suppress *Escovopsis* and, consequently, defend the ant cultivar [25]. Support for a benefit to the bacteria by their ant hosts includes (i) the presence of morphological modifications in or on the cuticle of the majority of attine ant genera for maintaining the bacteria, and/or specialized cuticular glands that apparently secrete nutrients to support bacterial growth [23], and (ii) vertical transmission of *Pseudonocardia* bacteria to new colonies by prospective queens in several attine ant genera [20]. The available evidence indicates that fungus-growing ants have associated with Actinobacteria for millions of years: morphological structures in the ant cuticle are present across most of the phylogenetic diversity of the Attini [23], and bacteria were visible on a Dominican amber specimen of *Trachymyrmex* sp. [26]. This apparent early origin of the actinobacterial symbionts fits with the predicted ancient functional requirements for the Actinobacteria for garden defence inferred by the early origin of *Escovopsis* parasitism in the symbiosis [13].

While patterns of specialization between ants and cultivar, and between *Escovopsis* and cultivar, have been established within this complex symbiosis, the specificity of the ant–*Pseudonocardia* association is less clear. Here, we combine phylogenetic and experimental approaches to explore the evolutionary history and specificity between fungus-growing ants and *Pseudonocardia* bacteria. Our phylogeny of attine ant-associated *Pseudonocardia* is based on 16S rDNA and elongation factor Tu sequences of bacteria isolated from 124 ant colonies, spanning most of the phylogenetic diversity of the symbiosis. Finally, we compare the degree of inhibition of *Escovopsis* and other fungi by ant-associated *Pseudonocardia*, free-living *Pseudonocardia* and free-living *Streptomyces* (a common genus of soil Actinobacteria).

2. MATERIAL AND METHODS

(a) *Bacterial strains*

We sampled actinobacterial symbionts associated with fungus-growing ants spanning the phylogenetic diversity of the attine tribe. Fungus-growing ant taxa sampled in this study included: (i) basal ant genera, designated as ‘lower’ attines, which include representative members of both the Palaeoattini (genera *Mycocepurus* and *Myrmicocrypta*) and the Neoattini (genera *Myzomela*, *Myzomelitopsis* and *Cyphomyrmex*); (ii) species of ‘Coral fungus agriculture’, members of the Palaeoattini in the genus *Apterostigma* that culture fungi in the family Pterulaceae; and (iii) the ‘higher’ attines, spanning the ‘Higher attine’ and ‘Leaf-cutter ant’ agricultural systems [11]. Actinobacterial isolations were performed using chitin agar plates containing antifungals (electronic supplementary material, figure S1–d); following described protocols (see [27])). Ant colonies were collected aseptically in the field, using flame-sterilized forceps and spoons. To reduce the risk of lateral transfer of symbionts between nests, isolations were typically conducted within 3–5 days of colony collection, and colonies were physically separated prior to sampling. To reduce the likelihood of including allochthonous bacteria in our study, we sampled 4–10 workers per individual colony and included only Actinobacteria morphotypes that were consistently associated with individual workers (see below and electronic supplementary material, figure S1) and between workers from individual nests. Actinobacteria are not present on the exoskeleton of *Atta* workers [23], so isolations were performed
Figure 1. (Caption overleaf.)
from workers that were vortexed or macerated in 500 µl of sterile water.

Our sampling yielded multiple genera of Actinobacteria, the vast majority of which was Pseudonocardia. Based on their abundance and presence throughout the diversity of the mutualism, and similar findings in previous studies [27–29], we included only Pseudonocardia in this study. We obtained Pseudonocardia symbionts from 124 ant colonies, spanning 19 different ant species and broad geographical locations (see electronic supplementary material, table S1).

Free-living Pseudonocardia and Streptomycetes were obtained from the US Agricultural Research Service (ARS) culture collection (Peoria, IL, USA) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

**DNA preparation and sequencing**

Pseudonocardia pure cultures were scraped-off from yeast malt-extract agar (YMEA) plates (approx. 500 mg) and suspended in 500 µl of cetyltrimethylammonium bromide (CTAB) buffer (10 ml 1 M Tris (pH 8.4), 5 ml 0.5 M EDTA (pH 8.0), 28 ml 5 M NaCl, 2 g CTAB, 57 ml ddH2O). Cells were ground and subjected to repeated cycles of freezing in liquid nitrogen and thawing at 65°C in a heat block. One volume of chloroform was added to 500 µl of sterile water.

One per cent isopropanol was carried out overnight at −20°C. After centrifugation, the DNA pellet was washed twice with 70 per cent ethanol and resuspended in 50 µl of TE/10 mM EDTA (pH 8.1). In most cases, total genomic DNA was cleaned with Elu-Quik purification kit (Whatman, NJ, USA) and aliquots were diluted (1:200) in ddH2O and used for amplification by PCR. Cycle parameters for PCR were adjusted depending on primer pairs.

PCR products were cleaned using commercial kits (Wizard PCR Prep DNA Purification System, Promega Corp., or Montage PCR extraction kit, Millipore, MA, USA). When multiple bands were present, these were separated on a low-melt agarose gel and subsequently cloned in a P-Gem T-Easy vector system (Promega Corp.). Sequencing reactions in both directions using Big Dye termination chemistry (Applied Biosystems, CA, USA) were performed at the UW Biotechnology Center. In some cases, amplification of bacterial DNA was achieved directly from whole-ant extraction. A single ant was crushed in a ceramic mortar cooled to −80°C with 1 ml of CTAB buffer, then extracted with 500 µl of chloroform. Overnight precipitation in 100 per cent isopropanol yielded enough DNA to clean and amplify target loci, and PCR product was either direct-sequenced or subjected to cloning using the P-Gem T-Easy vector (Promega Corp.).

**Phylogenetic analyses**

GenBank searches of raw sequences using BLAST were performed to verify Actinobacteria identity before incorporation into the analyses. After contig assembly in Sequencher 4.8 (GeneCodes, MI, USA), sequences were aligned in ClustalX [30] and manually edited in McClade v. 4.07 [31]. Sequences are deposited in GenBank, and taxon and accession numbers are listed in electronic supplementary material, table S1.

An initial phylogenetic analysis of 16S rDNA was performed to include as many described Pseudonocardia species as possible, covering most of the genus, and included 37 free-living Pseudonocardia, 29 Pseudonocardia isolates from representative attine ant species, and two Streptomycetes as an outgroup. Bayesian analyses were performed in MrBayes v. 3.1.2 [32], and all analyses employed one cold chain and three incrementally heated chains (T = 0.2). Four independent Markov chains were used in MrBayes searches, with two million generations each, were conducted. The initial 1000 generations from each run were discarded, and one in every 100 generations was sampled to calculate posterior probabilities for each branch. The general time-reversible model with Gamma distribution for rate variation (GTR + I + G) was used. Priors for the substitution rates were set to a flat distribution.

A second Bayesian analysis was conducted for the combined dataset (16S and EF Tu). The data were partitioned by gene and analysed under the same priors with variable site-specific rate model across the partitions. Model parameters for character state frequency, transition/transversion rate ratio and shape of the gamma distribution were unlinked across the partitions. The likelihood model was set to a general time-reversible model with a Gamma distribution for rate variation (GTR + I + G) based on results of a separate analysis of each gene using MrModelTest. Markov chain Monte Carlo search was set for 5'000'000 generations.
with one cold chain and three incrementally heated chains \((T = 0.015)\). Two separate runs with four chains each were performed saving every 500 trees. The consensus tree, generated using SumTrees [33] is the result from the combination of the 15 000 saved trees after burn-in.

A likelihood analysis was also conducted on the combined dataset using the GTR + I + G model. Four separate runs using default parameters were conducted in GARLI [34]. The best tree from each run was in concordance to the Bayesian tree. Likelihood bootstrap support values were generated from 1000 replicate runs and were summarized using SumTrees.

(d) Permutation test

We conducted a statistical evaluation to determine whether Pseudonocardia from the same ant agricultural system grouped more frequently together in our consensus phylogenetic two-gene analysis than expected by chance. The test involved assigning a membership in a particular group to each Pseudonocardia used, and asking whether these group assignments are randomly distributed on the phylogeny. To determine if Pseudonocardia are non-randomly associated with attine ant agricultural systems [11], we partitioned the leaves of the phylogeny into four groups that corresponded to these systems and free-living Pseudonocardia. As a measure of the diversity within each group, we computed the total sum of all branch lengths (TBL) in the pruned subtree containing only the leaves within each group, where the subtree is rooted at the most recent common ancestor of the group. Note that the sum of TBL values over groups is not the total sum of branch lengths in the original tree as some branches may be part of subtrees for multiple groups and some branches may not be included in the subtree of any group. Groups of low relative diversity will have small TBL values, whereas groups spread throughout the tree will have larger TBL values. The sum of TBL over groups is a measure of the overall correspondence between the groups and the phylogeny.

To determine a reference distribution for these TBL measures under a null hypothesis of no correspondence between the phylogeny and the groups, a permutation test was conducted. The groups of the leaves (excluding the outgroups) were permuted 10 000 times. For each permutation, the TBL values were calculated for each group and were also totalled, and both means and standard deviation (s.d.) were calculated for each group and for the total. As the reference distributions are observed to be approximately normal, the \(Z\)-test statistic \((Z = \frac{\text{TBL} - \text{mean}}{\text{s.d.}})\), which measures the distance of TBL for the actual groups from the means of the corresponding reference distributions in standard units are useful summaries for inference. The further \(Z\) is below zero, the stronger the clustering of the corresponding group relative to the clustering of a group of the same size selected at random from the tree.

(e) Bioassay analysis

In order to evaluate the antibiotic specificity of ant-associated Pseudonocardia against Escovopsis, we performed a Petri plate bioassay experiment. This bioassay evaluated the antibiosis of ant-associated Pseudonocardia, non-ant-associated (free-living) Pseudonocardia and free-living Streptomyces against diverse Escovopsis parasites and an assembly of other fungi. Pseudonocardia strains were isolated from 14 colonies spanning the attine ant phylogeny, including the ant species Apterostigma dentigerum, Cyphomyrmex muelleri, Mycetarotes parallelus, Myrmicocrypta sp., Trachymyrmex cornetzi, Acromyrmex niger, Acromyrmex hispidus fallax, Acromyrmex echinatior, Acromyrmex octospinosus and Atta colombica. Pseudonocardia strains were isolated using established methods ([27]; see above). Free-living Pseudonocardia were obtained from either ARS or DSMZ and non-ant-associated Streptomyces were isolated from environmental samples (soil and different plants) in Madison, WI, USA. Escovopsis isolates obtained from 10 fungus-growing ant colonies were revived from long-term glycerol storage of cultures. Non-fungus-growing ant-associated fungi were obtained from culture collections (Eurotium, Fusarium, Penicillium, Paecilomyces and Rhizopus from Carolina Biosciences, Burlington, NC, USA; and Beauvaria basianna and Matarhizium anisoplae from the Faculty of Life Sciences, Copenhagen, Denmark), as well as from environmental samples (Aspergillus, Isaria, Paecilomyces and Trichoderma). All fungi were maintained on potato dextrose agar containing antibacterials (streptomycin sulphate and penicillin-G; both at concentrations of 12.5 mg l\(^{-1}\)).

One replicate of each Actinobacterium–fungus combination was tested in a symbiont pairing bioassay experiment. The Actinobacterium was point inoculated in the centre of a YMEA plate without antibiotics and was grown for three weeks (diameter ca 1–1.5 cm), after which the fungus was inoculated at the edge of the plate. Plates were examined bi-weekly and when a clear zone of inhibition had formed in a given pairing (typically within two to three weeks after fungus inoculation), the minimum zone was measured (cf. [22]). We evaluated the extent of specificity in bacteria–fungus interactions using two-tailed \(t\)-tests in pairwise comparison of ant-associated Pseudonocardia, free-living Pseudonocardia and Streptomyces against both Escovopsis and other fungi. \(p\)-Values were adjusted using Bonferroni correction to reduce type I error when multiple comparisons are performed. \(F\)-tests were performed in order to establish whether individual \(t\)-tests should be performed with equal or unequal variances.

3. RESULTS AND DISCUSSION

(a) Abundant Pseudonocardia on fungus-growing ants

In our study, 105 of the ant colonies used were from four genera of fungus-growing ants: Apterostigma, Mycetarotes, Trachymyrmex and Acromyrmex. In each of these four genera, workers both carry an abundance of visible white ‘bloom’ of Actinobacteria on their cuticle (figure 1 and electronic supplementary material, figure S1a) and are large enough to facilitate targeted isolations of localized growth of Actinobacteria. In all of these ant colonies, multiple colony-forming units (CFUs) of morphologically similar Actinobacteria (see electronic supplementary material, figure S1b–d) were obtained from ant cuticles, and sequencing confirmed that these isolates were Pseudonocardia. We also isolated Pseudonocardia from the other five genera of fungus-growing ants examined: Myrmicocrypta, Mycocepurus, Mycetosoritis, Cyphomyrmex and Atta. One, three and four colonies of Mycetosoritis, Mycocepurus, Myrmicocrypta ants were sampled, respectively, and each ant colony yielded CFUs of morphologically similar Pseudonocardia isolates. For Cyphomyrmex, the Actinobacterium is visible on the cuticle of the propulelent plate of workers (see [23]), but our isolations from workers in this genus mostly yielded
no CFUs. In *Atta*, Actinobacteria are not present on the ant cuticle, and most isolation attempts were also unsuccessful. Nevertheless extensive plating of ant external washes and/or ant macerates yielded several *Pseudonocardia* isolates that grouped with other attine ant-associated *Pseudonocardia* from these attine genera. The inability to readily isolate *Pseudonocardia* from *Cyphomyrmex* workers indicates that the Actinobacteria associated with this genus are fastidious, as is common with microbes in other symbiotic systems. Our low success rate in isolating *Pseudonocardia* from *Atta* could likewise be owing to the symbiont not being readily culturable in these ants. Alternatively, unlike in *Cyphomyrmex*, Actinobacteria are not visible on *Atta* ants, so it is possible that the symbiont is not associated with this genus (see electronic supplementary material for further discussion on the association between *Atta* and *Pseudonocardia*).

In total, we identified *Pseudonocardia* symbionts associated with 124 colonies of fungus-growing ants, spanning 19 species and nine genera of attine ants. Our sampling covered most of the phylogenetic diversity of the tribe Attini, and much of the geographical distribution of the fungus-growing ant microbe mutualism (Argentina, Costa Rica, Ecuador, Panama, Peru and the USA; figure 1 and electronic supplementary material, figures S2 and S3). Our findings indicate that the visible actinobacterial symbiont of fungus-growing ants belongs to the genus *Pseudonocardia*. This finding is supported by various other studies. Cafaro & Currie [27] determined the generic placement of *Pseudonocardia* using ultrastructure examination of the bacteria directly on the cuticle of *Acromyrmex* workers. In addition, three population-level studies, representing 180 colonies from four fungus-growing ant species, support specific associations between fungus-growing ants and *Pseudonocardia* (34 colonies of *Acromyrmex octospinosus* and *A. echinatior*; [28]; 43 colonies in *Thachymyrmex septentrionalis*; [29]; and 103 colonies in *Apterostigma dentigerum*: E. J. Caldera 2010, personal communication). Thus, a total of more than 300 colonies of fungus-growing ants have been found to be associated with *Pseudonocardia;, all of which group phylogenetically within the six clades recognized here (figure 1), supporting that *Pseudonocardia* is a specific, and potentially the predominant, cuticular exosymbiont.

Other studies have isolated *Streptomyces* Actinobacteria from fungus-growing ant colonies (e.g. [35–37]; but note that they also isolated *Pseudonocardia*). This is not surprising given that *Streptomyces* is very common in soil [38], which is where the ants nest, and in plant material [39], which is integrated into their fungus garden. A few studies have argued against the *Pseudonocardia* generic placement of the exosymbiotic Actinobacteria in fungus-growing ants (i.e. [40,41]). Kost et al. [40] isolated 21 Actinobacteria strains from seven colonies of *Acromyrmex octospinosus*, two of which were identified as *Streptomyces*. Mueller et al. [41] isolated diverse genera of Actinobacteria from fungus gardens and workers of 10 fungus-growing ant colonies and from infrabuccal pellets from incipient *Atta texana* queens. Contrary to the view of these authors, the isolation of other Actinobacteria genera from fungus-growing ants does not negate the presence of exosymbiotic *Pseudonocardia* from ant workers, especially in the cases where, as in the latter study, isolations were not conducted from worker cuticles. However, given the ubiquitous nature of Actinobacteria in the environment of these ants, and the general recognition that isolation of a microbe alone is not sufficient evidence to establish a symbiotic association (see [42,43] for detailed discussion of challenges and requirements for establishing that associations are symbiotic), further evidence is necessary to support that *Streptomyces* bacteria are exosymbionts of the ants. Alternatively, other Actinobacteria may represent additional symbionts occupying different niches in the system, as has recently been revealed to be common (cf. [44–46]).

(b) Phylogenetic and specificity patterns in the ant–*Pseudonocardia* association

Given the frequency of the association between attine ants and *Pseudonocardia*, we focused on exploring patterns of specificity in this ant–bacteria symbiosis. *Pseudonocardia* isolated from the cuticles of fungus-growing ants do not constitute a monophyletic group, but rather occur in at least six main clades (five of which are well-supported), with free-living *Pseudonocardia* species occurring as basal or sister groups and in a few cases within the ant-associated *Pseudonocardia* clades (figure 1 and electronic supplementary material, figure S3). Nevertheless, a comparison of these major clades of ant-associated *Pseudonocardia* with the evolutionary history of attine ants reveals a significant degree of specificity, both at a broad evolutionary scale as well as at a finer phylogenetic level (figure 1). Permutation tests reveal that ant-associated *Pseudonocardia* strains are non-randomly associated with specific ant genera and with agricultural systems (figure 1 and table 1). In the ‘Lower attine agriculture’ system, all ant colonies were associated with sister clades II and III of our two-gene phylogeny of *Pseudonocardia* (figure 1 and table 1). In *Apterostigma* ants with ‘Coral fungus agriculture’, all but one of the strains (see below) grouped in two clades of *Pseudonocardia* (figure 1, clades IV and V and table 1). In the ant genera *Acromyrmex* and *Atta* (‘Leaf-cutter agriculture’), the vast majority of *Pseudonocardia* symbionts occur in the closely related clades IV and VI (figure 1). The least-specific pattern of *Pseudonocardia*–ant association occurs with members of the ant genus *Trachymyrmex* (non-leaf-cutter ‘Higher agriculture’ system). In this group, ants associate with strains of *Pseudonocardia* symbionts from four symbiont clades (I, III–VI), spanning much of the diversity of ant-associated *Pseudonocardia*. Nevertheless, this grouping was still non-random with respect to the full diversity of free-living and ant-associated bacteria in our analysis (figure 1 and table 1).

We also found evidence for ant–*Pseudonocardia* specialization at finer phylogenetic levels (figure 1). For example, all *Pseudonocardia* occurring in clade II were isolated from ant species in the two Palaeoattini genera *Myrmicocrypta* and *Mycocepurus*, with the exception of *Apterostigma* sp. 062 from Ecuador (note: it was not possible to confirm ant host species or cultivar type for this exception). Likewise, all sampled colonies of Neoattini ants with ‘Lower attine agriculture’ (i.e. *Cyphomyrmex*, *Mycetocryptis* and *Mycetarotes*) were found associated with *Pseudonocardia* that occur in clade III. Given that these five attine genera cultivate closely related fungi (‘Lower attine agriculture’ [11]), this reflects a potential pattern of specificity towards *Pseudonocardia* below the
Table 1. Permutation results from the test of non-random associations between *Pseudonocardia* isolates and attine agricultural systems. *n*, number of taxa in each group; TBL, total branch length; Z, standard score.

<table>
<thead>
<tr>
<th>group</th>
<th>n</th>
<th>TBL mean</th>
<th>s.d.</th>
<th>Z</th>
<th>p-valuea</th>
<th>p-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>free-living</td>
<td>16</td>
<td>0.725</td>
<td>0.448</td>
<td>0.101</td>
<td>2.75</td>
<td>0.997</td>
</tr>
<tr>
<td>lower attines</td>
<td>27</td>
<td>0.197</td>
<td>0.580</td>
<td>0.111</td>
<td>−3.46</td>
<td>2.7 × 10⁻⁴</td>
</tr>
<tr>
<td>coral-fungus growers</td>
<td>24</td>
<td>0.218</td>
<td>0.554</td>
<td>0.110</td>
<td>−3.06</td>
<td>1.1 × 10⁻³</td>
</tr>
<tr>
<td>higher attines</td>
<td>75</td>
<td>0.512</td>
<td>1.031</td>
<td>0.105</td>
<td>−4.94</td>
<td>3.9 × 10⁻⁷</td>
</tr>
<tr>
<td>leaf-cutter attines</td>
<td>46</td>
<td>0.206</td>
<td>0.788</td>
<td>0.118</td>
<td>−4.93</td>
<td>4.1 × 10⁻⁷</td>
</tr>
</tbody>
</table>

aOne-tailed probability based on numerical evaluation of the Z-score from 10 000 random permutations of the taxa within each group over the tree (website: http://www.fourmilab.ch/rpkp/experiments/analysis/zCalc.html). The test evaluates the hypothesis that the actual sum of branch lengths for the pruned subtree for the group is smaller than expected by chance, given the tree.

bOne-tailed probability, where the p-value is defined as the rank of the observed sum of branch lengths among 10 000 permutations of these taxa over the tree (p-value = rank_{observed}/(N_{permutations} + 1)).

level of agricultural system. In addition, some lineages of *Pseudonocardia* appear to be specific to individual ant species. For example, *Pseudonocardia* strains from clade I are exclusively associated with *Trachymyrmex zeteki*. Nevertheless, *Pseudonocardia* isolated from other colonies of *T. zeteki*, even from the same ant populations, appear in clades III and IV, indicating that a single ant species can associate with more than one *Pseudonocardia* lineage. Similarly, *Pseudonocardia* symbionts of *T. cornetti* also span multiple clades (IV–VI). A finding of specificity at finer phylogenetic levels is also supported by permutation tests of non-random patterns of *Pseudonocardia* association by ant genus (see electronic supplementary material, table S2). This pattern of specificity suggests that exchanges of *Pseudonocardia* between colonies of fungus-growing ants may be restricted by ant-bacterial incompatibility, potentially mediated by (i) glandular secretions in the ants [23] and/or (ii) behavioural recognition of *Pseudonocardia* by worker (known in *Acromyrmex*: [47]).

We explored explanations other than biological specificity for the non-random associations between *Pseudonocardia* and attine agricultural systems. Two possible explanations are that (i) free-living *Pseudonocardia* strains are themselves geographically subdivided, with sampling of different attine groups from different geographical regions spuriously generating non-random patterns; and (ii) different researchers generated non-random patterns by sampling ants in ways that somehow biased *Pseudonocardia* phylogenetic patterns. We repeated the permutation test for samples collected in the two most intensely sampled geographical regions (Panama or Argentina), which produced results essentially equivalent to the main analyses (electronic supplementary material, table S3). Similarly, we repeated the permutation test for researcher sampling bias for leaf-cutter and coral fungi agricultural systems, partitioned by researchers for which there was sufficient replication. Also, this analyses showed no evidence for non-random associations between *Pseudonocardia* and researcher within the agricultural systems (electronic supplementary material, table S4).

(c) **Experimental patterns in Pseudonocardia antibiosis against Escovopsis**

Our symbiont bioassay experiments, pairing ant-associated *Pseudonocardia* strains with *Escovopsis* and other fungi, revealed abundant variation in the suppression of fungal growth (electronic supplementary material, figure S4). In general, *Escovopsis* strains were strongly inhibited by Actinobacteria-derived compounds, including both ant-associated and free-living strains. Further, *Escovopsis* was more susceptible to the antibiotics produced by Actinobacteria than other general fungi tested in this study (figure 2 and electronic supplementary material, figure S4). The capacity of some free-living *Pseudonocardia* and *Streptomycetes* species to inhibit *Escovopsis* is not surprising, as many Actinobacteria produce secondary metabolites with antifungal activities [48,49]. Ant-associated *Pseudonocardia* were significantly more effective in the inhibition of *Escovopsis*, when...
compared with the inhibition of Escovopsis by either free-living Pseudonocardia or Streptomyces (t-test, \(p_{\text{adj}} < 0.0002\); figure 2 and electronic supplementary material, table S5). Free-living Pseudonocardia species varied in their degree of Escovopsis inhibition, and were comparable to Streptomyces in their inhibition of Escovopsis (\(p_{\text{adj}} = 0.9434\); figure 2 and electronic supplementary material, figure S4 and table S5). These findings not only indicate that Escovopsis spp. are generally more susceptible to inhibition by Actinobacteria, but that they are more strongly inhibited by ant-associated isolates than free-living Pseudonocardia and Streptomyces. Further, the variation in the inhibition profiles suggests that different ant-associated Pseudonocardia strains either produce different compounds, or secrete the same suite of compounds but in different concentrations (see electronic supplementary material, figure S4). This underlying variation is known to be important in Acromyrmex, where infection with Escovopsis resistant to Pseudonocardia-derived antibiotics induces increased garden biomass loss when compared to infections with susceptible strains [22].

4. CONCLUSIONS

Many genera of fungus-growing ants have visible growth of exosymbiotic Actinobacteria on their cuticle [20,23]. The presence of elaborate cuticular crypts and specialized exocrine glands for maintaining cuticular Actinobacteria symbionts supports that this is an ancient association [23]; however, the species strain specificity of Pseudonocardia with ant hosts has remained unclear. Through examination of the broad phylogenetic patterns of host associations between the ants and Pseudonocardia spp., we reveal that ant-associated Pseudonocardia share a significantly greater degree of specificity with their ant hosts than would be expected by chance. Specifically, we identified patterns involving related groups of ants typically associating with specific clades of Pseudonocardia.

However, the ant–Pseudonocardia association is also shaped by lateral symbiont transfers between ant colonies, as well as acquisitions of novel environmental Pseudonocardia strains. Further, we find that ant-associated Pseudonocardia are significantly better at inhibiting Escovopsis than are either free-living Pseudonocardia or Streptomyces. Specialized host–symbiont relationships appear to be frequently maintained through the evolution of mechanisms that ensure specificity [3–5], and future work on the ant–Pseudonocardia symbiosis should explore the mechanisms that have evolved to result in the formation and maintenance of this host–symbiont association.

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