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Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants

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Abstract

Acromyrmex **leaf-cutting ants maintain two highly specialized, vertically transmitted mutualistic ectosymbionts: basidiomycete fungi that are cultivated for food in underground gardens and actinomycete** ***Pseudonocardia*** **bacteria that are reared on the cuticle to produce antibiotics that suppress the growth of** ***Escovopsis*** **parasites of the fungus garden. Mutualism stability has been hypothesized to benefit from genetic uniformity of symbionts, as multiple coexisting strains are expected to compete and, thus, reduce the benefit of the symbiosis. However, the** ***Pseudonocardia*** **symbionts are likely to be involved in Red-Queen-like antagonistic co-evolution with** ***Escovopsis*** **so that multiple strains per host might be favoured by selection provided the cost of competition between bacterial strains is low. We examined the genetic uniformity of the** ***Pseudonocardia*** **symbionts of two sympatric species of** ***Acromyrmex*** **ants by comparing partial sequences of the nuclear Elongation Factor-Tu gene. We find no genetic variation in** ***Pseudonocardia*** **symbionts among nest mate workers, neither in** ***Acromyrmex octospinosus*****, where colonies are founded by a single queen, nor in** ***Acromyrmex echinatior*****, where mixing of bacterial lineages might happen when unrelated queens cofound a colony. We further show that the two ant species maintain the same pool of** ***Pseudonocardia*** **symbionts, indicating that horizontal transmission occasionally occurs, and that this pool consists of two distinct clades of closely related** ***Pseudonocardia*** **strains. Our finding that individual colonies cultivate a single actinomycete strain is in agreement with predictions from evolutionary theory on host–symbiont conflict over symbiont mixing, but indicates that there may be constraints on the effectiveness of the bacterial symbionts on an evolutionary timescale.**

Keywords: antibiotics, conflict, *Escovopsis*, mutualism, parasitism, *Pseudonocardia*

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Introduction

Mutualistic interactions will only be evolutionarily stable when the benefits continue to be returned to the donor or to its close relatives (Frank 1994). Kinship tends to fulfil this condition when interacting partners are members of the same breeding population. However, in interspecific interactions, competition and exploitation are likely to prevail, unless sanctions against cheating can evolve (Trivers 1971; Frank 1996, West *et al*. 2002; Frank 2003; Kiers *et al*. 2003) so that conflict is suppressed or regulated without major costs for the partners involved (Frank 1996; Herre *et al*. 1999; Bot *et al*. 2001). Vertical transmission of symbionts is a very efficient mechanism to reduce reproductive conflict between host and symbionts. However, despite the benefits obtained from the association with the host, a vertically transmitted symbiont is expected to remain under selection for at least some horizontal dispersal, because of increasing fitness costs of competition between close kin (Hamilton & May 1977). Frank (1996, 1997) referred to this phenomenon as

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'disruptive kin selection' or the 'paradox' of kin selection, and subsequent studies have obtained an explicit understanding of the opposing forces of kin selection and sibling competition for the maintenance of cooperation (Taylor & Irwin 2000; West *et al*. 2001; Foster 2004; Griffin *et al*. 2004).

The hosts of vertically transmitted endosymbionts should be highly effective in preventing horizontal dispersal, as multiple barriers of cell walls and surface tissue severely restrict symbiont mobility. However, these barriers are far from absolute, as recent studies of *Wolbachia* have shown (Vavre *et al*. 1999; Tsutsui *et al*. 2003; Van Borm *et al*. 2003). Vertical transmission should thus be even harder to enforce upon mutualistic ectosymbionts, both because these symbionts have better possibilities to avoid host containment measures, and because horizontally transmitted propagules have higher probabilities of infecting a similar host–symbiont association close by. Given the relative likelihood of horizontal symbiont transmission and the potential damage of infections with alien symbionts to the fitness of hosts, theory predicts that hosts of ectosymbionts should be under strong selection to recognize symbiont mixing and eliminate infections with competing strains that would otherwise trigger virulent reactions by their resident symbiont (Frank 1996, 1997, 2003).

Acromyrmex (Formicidae: Attini) leaf-cutting ants culture mutualistic fungi in the family *Lepiotaceae* (Agaricales: Basidiomycota) (Weber 1966, 1972; Chapela *et al*. 1994), and in return the fungi serve as the main food source for the ants (Möller 1893). The ants provision the fungus with fresh plant material and protect it against competitors and pathogens (Bass & Cherrett 1994; North *et al*. 1997; Currie 2001a; Currie & Stuart 2001). The clonal fungus is vertically transmitted by the colony-founding gynes (winged prospective queens) (Hölldobler & Wilson 1990; after von Ihering 1898; Autuori 1956), but phylogenetic studies indicate that, as expected, horizontal exchanges have occurred repeatedly (Mueller *et al*. 1998; Bot *et al*. 2001; Green *et al*. 2002). However, both direct fungal incompatibility (Hansen *et al*. 1993; Poulsen & Boomsma 2005) and indirect ant behavioural incompatibility (Bot *et al*. 2001; Poulsen & Boomsma 2005) ensure that colonies continue to maintain a single lineage of fungus and that mixing of symbionts in mature colonies normally fails.

The recent discovery of a third mutualist in the ant–fungus symbiosis, an ectosymbiotic actinomycete bacterium in the genus *Pseudonocardia* (Pseudonocardiaceae, Actinomycetales) (Currie *et al*. 1999b, 2003b; Cafaro & Currie, 2005), provides interesting opportunities to further explore the applicability of host–symbiont conflict theory in the fungus-growing ant model system. This bacterium produces antibiotics that significantly inhibit microfungal *Escovopsis* (Ascomycota: anamorphic Hypocreales) parasites that attack the mutualistic fungus (Currie *et al*. 1999a, 2003a; Currie 2001b). The ants provide nutrients to the bacterium, a maintenance practice that carries a cost equivalent to *c*. 10–15% of the basic metabolic rate of major ant workers when they are completely covered by the bacterium (Poulsen *et al*. 2003). Also this bacterial symbiont is vertically transmitted via the cuticle of dispersing gynes (Currie *et al*. 1999b).

Where the maintenance of single symbiont strains of fungi in mature fungus gardens unambiguously corresponds to theoretical expectations (Bot *et al*. 2001; Frank 2003; Poulsen & Boomsma 2005), there is no single a priori prediction for the within-colony genetic diversity of the *Pseudonocardia* symbiont. In contrast to the fungus-garden mutualist, the actinomycete is almost certainly involved in a Red-Queen-like evolutionary arms race with its specific, horizontally transmitted, *Escovopsis* enemy (Currie *et al*. 1999a, 2003a; Currie 2001a). According to this hypothesis, the maintenance of high levels of genetic variation would increase the ability in a host to continuously evolve, so that its resistance mechanisms stay tuned to virulence innovations of presumably faster-evolving parasites (Howard & Lively 2002). This implies that benefits of genetic exchange by lateral gene transfer and recombination after mixing of bacterial lineages could potentially compensate for short-term productivity costs to the host of occasional 'infection' with other actinomycete strains (Poulsen *et al*. 2003). The latter scenario would be similar to the maintenance of costly multiple mating in *Acromyrmex* ants, which allows for a genetically diverse worker force with a more robust collective defence against disease (Hughes & Boomsma 2004; Sumner *et al*. 2004a).

The present study examines which of these alternative scenarios applies in two sympatric species of *Acromyrmex* leaf-cutting ants, *Acromyrmex echinatior* and *Acromyrmex octospinosus*, from Panama. We use sequencing data of the *Pseudonocardia* symbionts of 35 colonies to establish (i) whether the two ant species share the same pool of symbionts or are associated with genetically different clades of *Pseudonocardia*; (ii) whether there is significant genetic variation among *Pseudonocardia* strains across colonies of the same ant species; (iii) whether the *Pseudonocardia* symbiont shows any genetic diversity within a single colony of host ants; (iv) whether multiple symbiont strains are particularly likely in ant colonies with more than one queen, which happens occasionally in *A. echinatior* (Bekkevold *et al*. 1999), but not in *A. octospinosus* (Boomsma *et al*. 1999).

Materials and methods

Material used

Our ant samples came from a total of 16 colonies of *Acromyrmex echinatior* and 18 colonies of *A. octospinosus*. Part of these were collected by Danish team members in

Gamboa, Panama, from 1994 to 2002 and comprised seven polygynous (more than a single mother queen) colonies of *A. echinatior* (*A. ech.* colony numbers: 12, 20, 26, 43, 47, 134, and 168), eight monogynous (single queen) colonies of *A. echinatior* (*A. ech.* colony numbers: 10, 24, 33, 44, 48, 112, 113, and 174), and nine (always monogynous) colonies of *A. octospinosus* (*A. oct.* colony numbers: 13a, 21, 31, 34, 35, 38, 101, 117, and 170). The samples used were major workers that had been stored in 96% ethanol since collection in the field. The remaining material was collected by American team members and comprised nine monogynous colonies of *A. octospinosus* (*A. oct.* colony numbers 1, 7, 13b, 15a, 15b, 25, 30, 167 and 168), and one monogynous colony of *A. echinatior* (*A. ech.* colony number 219). They were collected at the same site in 2001, 2002, and 2003 and had been maintained at the University of Kansas until they were sampled for the present study. From these colonies we used either live ants, carrying a partial or complete cover of the actinomycete bacterium, or pure cultures of the bacterium in 1–4 replicates (one exceptional case had 24 replicates). These cultures were obtained by aseptically scraping bacteria off the ant cuticle and transferring the inoculum to chitin agar plates containing nystatin (10 000 units/mL). After bacterial growth (*c*. 3–5 weeks), colonies were subcultured on to Czapek yeast autolysate agar (Frisvad & Filtenborg 1989).

DNA extraction

In addition to extractions from scrapings of pure culture plates, ant tissue samples from either live ants or alcohol material were used. Ant tissue was placed in 500 µL CTAB [10 mL 1 м Tris (pH 8.4) + 5 mL 0.5 м EDTA (pH 8) + 28 mL 5 м NaCl + 2 g CTAB + 57 mL ddH$_2$O, stirred and filtered through a 0.22 µм filter]. Before placing the tissue in CTAB, ant material was left at room temperature until the alcohol had evaporated. The bacterial and ant tissue were ground in the CTAB, and subsequently subjected to three freeze–thaw cycles in order to release DNA to the solution. Thereafter, 500 µL chloroform was added and the suspension was vortexed vigorously. The samples were then spun at 16 000 *g* for 15 min, and the aqueous phase (including the DNA) was recovered from the tubes without distorting the tissue and chloroform phases. Following this, 400 µL of cold (–20 °C) 100% isopropanol was added, after which the tubes were inverted and left overnight at –20 °C. The following day, the samples were spun at 16 000 *g* for 20 min, and the liquid phase was removed without distorting the pellet in the tube. The pellet was gently washed with 500 µL 70% ethanol, spun down at 16 000 *g* for 3 min, after which the alcohol was discarded and the pellet was dried using a vacuum drier (Labconco, CentriVap Concentrator). Thereafter, the pellet was gently resuspended in either 30 µL 0.2 µm filtered and autoclaved water, or in 30 µL 1/10 TE (1 mL 1 м Tris-HCl buffer + 100 µL 0.1 м EDTA + 98.9 mL ddH20, adjusted to pH 8.0–8.5 and filtered through a 0.2 µм filter). Extracts were subsequently stored at –20 °C.

DNA amplification, PCR purification, and sequencing

We amplified and sequenced part of the nuclear gene Elongation Factor-Tu (*EF-Tu*), which codes for a GTP binding protein that plays a central role in protein synthesis, and is considered appropriate for phylogenetic analyses (Ke *et al*. 2000 and references therein). Amplifications were performed using either undiluted or 1/20 diluted genomic DNA for extractions from ants (where the concentrations of DNA in the extractions were low), and 1/100 diluted genomic DNA for extractions from plated actinomycetes (where the initial concentration of DNA was too high to perform polymerase chain reaction). The primer combinations used to obtain the sequences were either 1F (5′-GGCTTC-GGCGTTCGACAT-3′) × 1048R (Ludwig *et al*. 1993) or 52F (Ludwig *et al*. 1993) × 920R (5′-GCCGCCCTCATCCTT-GCCC-3′), both combinations being specific to *Pseudonocardiaceae*, as 1F and 920R were new primers designed on the basis of sequences obtained from pure cultured *Pseudonocardia* bacteria isolated from *Acromyrmex* ants (Cafaro & Currie, unpublished). Twenty-microlitre PCRs (Kocher *et al*. 1989), containing 0.3 µL *Taq* polymerase, 10 µL 2 × Mastermix (consisting of 3.4 µL H$_2$O, 3.6 µL dNTP, 2.0 µL buffer, and 0.5 µL of each primer), 2–10 µL sample, and 0–8 µL H$_2$O, were performed using an MJ Research PTC-200 PCR machine with the following program: 96 °C for 2.5 min, 40 cycles of 94 °C for 45 s, 56 °C for 50 s, 72 °C for 2 min and a final extension period of 10 min at 72 °C. Following PCR amplification and provided a single band was observed on a 1% agarose gel in 1× TAE buffer, PCR purification of 2–6 combined PCR products was performed using the Wizard® PCR Preps DNA Purification System (Promega) protocol, which was modified so that dilutions were carried out in only 20 µL of autoclaved and 0.2 µм filtered water. When multiple products were present, the bands were separated by gel electrophoresis (Fisher Scientific) and purified using QIAquick Gel Extraction Kit (QIAGEN). Sequencing reactions were performed using CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter), cleaned using CleanSEQ™ reaction Clean-Up (Agencourt Bioscience) and loaded on to a CEQ™ 8000 Genetic Analysis System (Beckman Coulter).

Phylogenetic analysis

The nucleotide sequences obtained were aligned using CLUSTAL _X version 1.81 (Thompson *et al*. 1997), MACCLADE 4.06 OS X (Sinauer Associates), and SEQUENCHER 4.1 (Gene

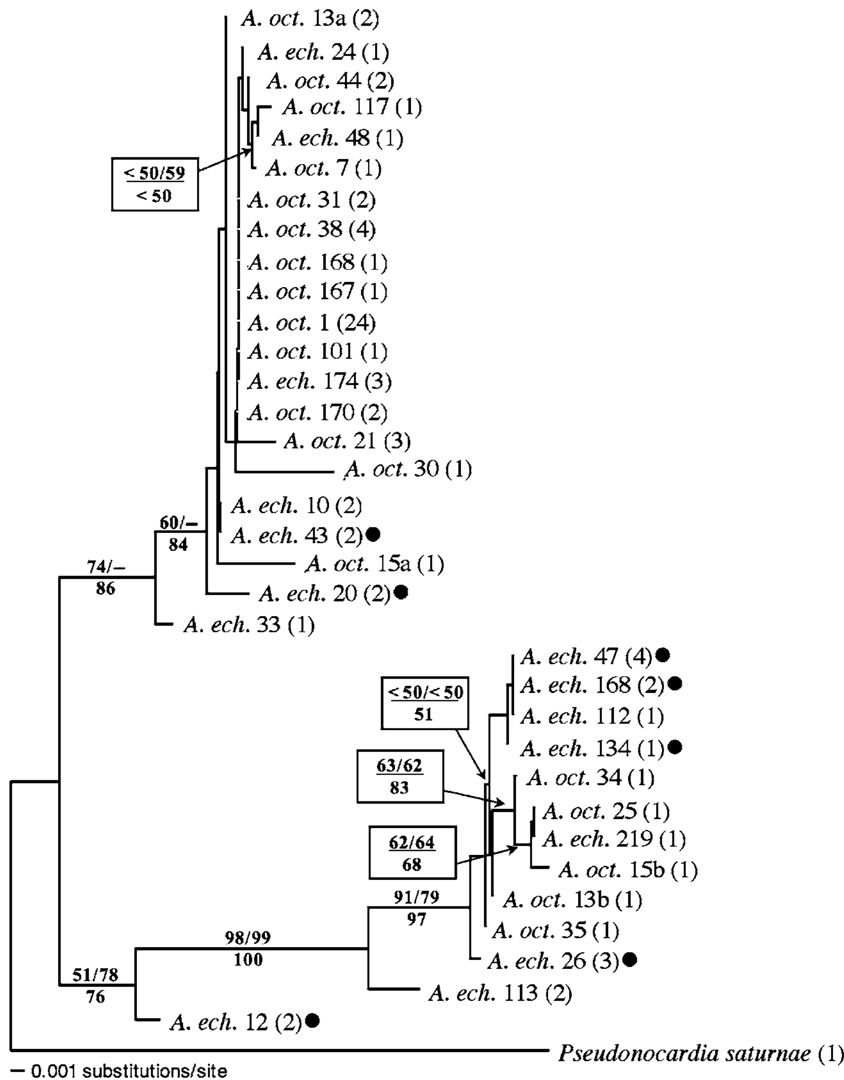

Fig. 1 A phylogenetic neighbour-joining consensus tree of the *Pseudonocardia* bacteria associated with Panamanian *Acromyrmex echinatior* (*A. ech.*) and *A. octospinosus* (*A. oct.*) obtained from heuristic searches based on the 763 bases in the Elongation Factor region. The number of sequences obtained from individual ants from within each colony is given in brackets and depended on the number of successful PCR and sequencing reactions. Black dots indicate colonies of *A. echinatior* having more than one queen. Branch values indicate bootstrap support of 1000 pseudoreplicates under MP (top left), ML (top right) and NJ (bottom) conditions, respectively. Bootstrap values exceeding 50 are given, and '–' indicates that branches are missing in the given bootstrap analysis.

Codes Corporation). All sequences obtained in this study are deposited with GenBank under Accession nos DQ098118–51. A sequence of *Pseudocardia saturnae* was obtained (GenBank Accession no. DQ098152) and used as the outgroup in the phylogenetic analyses (cf. the phylogenetic placement by Cafaro & Currie, 2005), which were performed in PAUP* 4.0b10 (Swofford 2002), inferring a neighbour-joining (NJ) phylogeny (Fig. 1). Node support was assessed under NJ, maximum-parsimony (MP), and maximum-likelihood (ML) conditions (see, e.g. Maynard Smith 2002), via heuristic searches (stepwise addition and TBR branch swapping). Characters were all treated as unordered and gaps as missing data. Bootstrap support was calculated for internal branches after 1000 pseudoreplicates for all tests run. For the ML analysis, we used the best fit model of sequence evolution estimated using MRMODELTEST version 2.1 (Nylander 2002), which showed that this model had equal substitution rates among bases (F81), among site variation in substitution rates distributed according to a gamma distribution ($\gamma = 0.9826$) and a proportion of invariable sites ($I = 0.7420$).

Results

The NJ tree obtained from the analysis of the 763 bases in the elongation factor region showed that there are two major clades of actinomycetes and that these clades are maintained as symbionts by both ant species (Fig. 1). The separation in two clades of symbionts has strong bootstrap support and is consistent between the three analyses. Within-clades, however, there are generally low bootstrap values and genetic diversity is very low, so that many colonies seem to rear essentially the same actinomycete strain. In the MP analysis, 86 characters were parsimony-informative and a single best tree was obtained with the length 599, a CI of 0.918, and a retention index (RI) of 0.894. This analysis differed only slightly from the NJ analysis, and changes never occurred between the two major clades.

Overall, the ML analysis gave the same result, except that one colony (*A. ech.* 33) grouped as the most basal node of the ingroup, but with low node support (Fig. 1).

We did not find any genetic differences among the multiple actinomycete sequences obtained from replicate workers (1–4 and 24) per colony (Fig. 1) in either monogynous or polygynous colonies. Based on this fact, and the present sample size, we calculated the probability that our study would have detected a potential second strain within a given colony. We used a general binomial power-test assuming that: (i) if there was more than one strain per colony, the number was two; (ii) if a colony maintained more than one strain, the frequency of the dominant and the alternative strain was the same in all colonies; (iii) the lack of within-colony variation in *EF-Tu* sequences obtained was representative for the entire genome of the *Pseudonocardia* symbiont. The test thus estimated the expected maximum frequency of a hypothetical second strain (see Poulsen & Boomsma 2005 for the precise formula). We performed the test with a very high power ($R^2 = 0.90$) and found that our present data allowed the conclusion that the maximum frequency of an additional second strain within an average colony would be 3.6%.

Discussion

Horizontal transmission and the maintenance of genetic variation

The two *Acromyrmex* ant species examined in this study are phylogenetically distinct (Sumner *et al*. 2004b). This implies that exclusive vertical transmission of the *Pseudonocardia* symbiont between ant generations should have resulted in two distinct clades of symbionts, each associated with a single ant species. However, we find that the bacterial symbionts are shared. This is consistent with the pattern found for the fungal symbionts of these ant species at the same site (Bot *et al*. 2001; Poulsen & Boomsma 2005), indicating that, at least over evolutionary time, occasional horizontal transmission is a fundamental trait that characterizes the entire symbiosis between farming ants, their fungal crops, and their *Pseudonocardia* bacteria, in spite of the default vertical transmission mode for both mutualistic symbionts.

Although phylogenetic incongruence between clades of ants and symbionts can be explained by events of horizontal transmission, there is no straightforward explanation for the surprising finding of two highly distinct clades of the bacterial symbiont. As the Central American *Acromyrmex* species form a distinct clade within a larger sample containing about half of the described species of the genus (Sumner *et al*. 2004b), it could be that some major clades of *Acromyrmex* have evolved allopatrically. We thus hypothesize that the *Pseudonocardia* symbionts associated with early Central American *Acromyrmex* may have diverged this way. After sympatry was restored (possibly after the Isthmus of Panama closed), the two *Pseudonocardia* clades would then have spread by horizontal transmission and come to coexist in extant Panamanian populations of *Acromyrmex* ant species.

Within each of the two distinct clades of actinomycete symbionts we found very limited or no genetic variation between the bacterial strains cultivated by separate colonies. This seems to be distinctly different from the fungal symbionts, which show more regular phylogenetic trees with many clades and subclades shared between these two ant species (Bot *et al*. 2001). Even though part of this difference may be due to the fungal analyses being based on amplified fragment length polymorphism (AFLP) genotyping and the *Pseudonocardia* analysis being based on sequences of a specific gene, it seems likely that the available genetic variation of actinomycete symbionts is lower than the genetic variation of the fungal symbionts.

One explanation for this might be that the actinomycete is physically more tightly associated with the ants (Currie *et al*. 1999b, 2003b), which may restrict horizontal transfers and the maintenance of variation via genetic recombination between strains. Although actinomycete inocula have been found in fungus garden material (Poulsen *et al*. 2003), this would not necessarily lead to more frequent simultaneous horizontal transmission of the fungus and the bacterium, because colonies may be more likely to lose their resident fungal symbiont than their resident bacterial symbiont. This could imply that bacterial inocula are less likely to get established in neighbouring colonies because of competitive conflict with the resident symbiont (see next section).

Single strains of symbiont per colony

Despite sequencing bacteria from up to 24 ants per colony, we never found different sequences within a given colony, indicating that the *Pseudonocardia* bacteria are present as single strains. This lack of sequence divergence within colonies could be due to limited variation in the Elongation Factor region analysed (nondetection error) or to estimation errors of limited sample size within colonies (nonsampling error) (cf. similar aspects of paternity analysis in social insect societies, e.g. Pedersen & Boomsma 1999). Regarding nondetection errors, it is clear that the use of the *EF-Tu* gene gives a much higher resolution than the 16S-rRNA region, which has traditionally been used to resolve bacterial phylogenies (e.g. Warwick *et al*. 1994; Ludwig & Schleifer 1999; Lee *et al*. 2000; Cafaro & Currie, 2005), and which is not variable enough to resolve between-colony genetic differences in actinomycete symbionts (M. Poulsen, unpublished). To completely establish that the actinomycetes associated with individual ant

colonies occur as single genetic strain requires more variable genetic markers. However, our single-gene approach did allow us to separate the two major clades of symbionts, so that nondetection error at this level should have been negligible. Therefore, if multiple strains are present within nests they must be genetically very closely related.

We estimated the nonsampling error by investigating the power of our conclusion of a single strain per nest with a general binomial test. Setting the required power at a high level (90%), this test iteratively calculated the probability of detecting double-strain colonies given the sample sizes within colonies that were available, showing that frequencies of an alternative strain as low as 3.6% would have been detected. Thus, even though we cannot exclude that additional strains could occur at very low frequencies, we can confidently exclude that the ants actively pursue the maintenance of genetically diverse cultures of actinomycetes in balanced frequencies.

One possible reason for the maintenance of single-strain bacterial symbionts could be that the ants rarely, if ever, encounter additional actinomycete strains. This seems, however, unlikely as unrelated, supposedly drifting, worker genotypes have been observed in low frequencies in one of the species (Boomsma *et al*. 1999). Given the roughly equal frequencies of the two major *Pseudonocardia* clades, this implies that approximately half of such random drifting workers would carry a different bacterial strain. A more likely explanation for single symbiont cultures is that colonies are constrained by costs associated with the rearing of multiple genetically distinct actinomycete strains, because the direct (short-term) costs of competition between these symbionts would be significant. Such costly incompatibilities between genetically different fungus clones originating from different *Acromyrmex* colonies have been demonstrated (Bot *et al*. 2001; Poulsen & Boomsma 2005) and similar incompatibilities have been observed in pilot experiments rearing the strains of the two major *Pseudonocardia* clades on the same medium (M. Poulsen, J. J. Boomsma, & C. R. Currie, unpublished). Any repression of such conflict by avoiding symbiont mixing would have immediate benefits for the mutualistic association and would maintain selection on host ants to eliminate secondary acquired strains (cf. Frank 1996, 2003).

This study and previous work allow us to conclude that the symbiosis between the two *Acromyrmex* species and their respective fungal and bacterial mutualists is highly skewed in terms of genetic diversity. The ants are known to have a high within-colony genetic diversity because queens mate with many males (Boomsma *et al*. 1999; Villesen *et al*. 2002; Sumner *et al*. 2004a), but their symbionts are both vertically and clonally propagated and lack any genetic diversity within colonies even though horizontal transmission happens occasionally.

The dynamics of host–symbiont co-evolution

All data collected so far (Currie *et al*. 1999b; Currie 2001a; this study) suggest that the *Pseudonocardia* symbionts have been domesticated by the attine ants shortly after the evolution of fungus farming, and that they have co-evolved with the ants and the local strains of *Escovopsis* that attack their fungus gardens. The co-evolution between *Escovopsis* and the *Pseudonocardia* symbionts may be characterized by Red-Queen-like frequency-dependent selection favouring rare genotypes. This should favour a complex of frequently recombining bacterial genotypes within each ant colony, providing a more robust defence against multiple *Escovopsis* infections during the typical long lifespans of *Acromyrmex* colonies (more than 10 years for laboratory colonies: Weber 1972) than a monoculture of the bacterium (cf. Poulsen *et al*. 2003). A benefit for such genetic variation among the antibiotic-producing *Pseudonocardia* symbionts seems likely, given the ample genetic variation that can be maintained in its 'opponent', the horizontally transmitted *Escovopsis* parasite (Currie 2001a; Currie *et al*. 2003a, c; cf. Poulsen *et al*. 2003). Diseases with this kind of evolutionary potential have been hypothesized to be a major selection force for the maintenance of genetic variation among the host ants themselves (Hamilton 1987; Sherman *et al*. 1988), and recent work has indeed shown that genetically diverse groups of *Acromyrmex* workers are less sensitive to infections with insect pathogenic fungi (Hughes & Boomsma 2004). Finally, human pharmaceuticals with antibiotic properties tend to elicit resistance in their target pathogens within at most a few decades (Baquero & Blázquez 1997; Levin & Anderson 1999), but nothing like this seems to have happened in the ancient co-evolutionary interaction between *Pseudonocardia* and *Escovopsis*.

Although there seems little doubt that the antibiotics-producing symbionts with low genetic diversity continue to provide potent antibiotic defences against *Escovopsis* (Currie *et al*. 1999b, 2003a), it is also reasonable to hypothesize that the effectiveness of the ant–*Pseudonocardia* symbiosis is constrained by the symbiont-imposed avoidance of symbiont mixing within colonies. Small immediate costs of competition are thus enough to select for ant behaviours to avoid symbiont mixing, even though the long-term benefits of increased resistance would potentially have been large (Frank 1996, 2003).

The phylogenetic analysis showed that colonies of *A. echinatior* having two or more functional mother queens do not display any within-colony diversity in the *Pseudonocardia* symbiont, consistent with the expectation that such coexistence of symbiont strains cannot be maintained. A similar result was found for the fungal symbiont, which also occurs as a single strain in colonies with multiple queens (Poulsen & Boomsma 2005). This result would be

trivial if the queens were all close maternal relatives, but empirical results indicate that this is unlikely to be the case in *Acromyrmex* species (Rissing *et al*. 1989; S. R. Sumner, W. O. H. Hughes & J. J. Boomsma, unpublished). Alternatively, it could imply that cofounding queens only associate when they have the same symbiont or that all but one symbiont strain is lost shortly after cofounding.

Thus, despite the very different biological roles of the fungal and bacterial symbiont associated with the same ant host, the selection forces against mixing of symbiont lineages within colonies appear similar (Bot *et al*. 2001; Poulsen & Boomsma 2005; this study) and in line with current theory on within-host symbiont conflict and the short-term evolutionary stabilization of mutualistic interactions (Frank 1996, 2003; Herre *et al*. 1999). The mechanisms that realize these convergent adaptations may be very different and are still largely unknown. The specialized interactions between fungus-growing ants and their two very different mutualistic ectosymbionts will thus continue to offer unique opportunities to test theories on the ecological efficiency and evolutionary stability of symbiotic interactions (Mueller *et al*. 2001; Mueller 2002; Currie *et al*. 2003c; Poulsen & Boomsma 2005).

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