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Diversity of entomopathogenic fungi near leaf-cutting ant nests in a neotropical forest, with particular reference to *Metarhizium anisopliae* var. *anisopliae*

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Abstract

We investigated the prevalence of entomopathogenic fungi associated with leaf-cutting ant colonies in a small area of tropical forest in Panama. There was a high abundance of *Metarhizium anisopliae* var. *anisopliae* near the colonies. *Beauveria bassiana* was also detected in the soil, *Aspergillus flavus* in dump material, and six *Camponotus atriceps* ants were found infected with *Cordyceps* sp.. Based on a partial sequence of the IGS region, almost all of the *M. anisopliae* var. *anisopliae* isolates fell within one of the three main clades of *M. anisopliae* var. *anisopliae*, but with there still being considerable diversity within this clade. The vast majority of leaf-cutting ants collected were not infected by any entomopathogenic fungi. While leaf-cutting ants at this site must, therefore, regularly come into contact with a diversity of entomopathogenic fungi, they do not appear to be normally infected by them.

Keywords: *Metarhizium*; *Atta*; *Acromyrmex*; Neotropics; Entomopathogen; Parasite; Diversity

1. Introduction

Social insects would be expected to be particularly prone to pathogens because of their group-living lifestyle, with its concomitantly high rate of interactions between individuals and the high relatedness of the interacting individuals (Alexander, 1974). However, relatively few parasites are known from social insects, in spite of their abundance and ecological dominance (Schmid-Hempel, 1998). This may relate to a genuine paucity, possibly resulting from a reduction in intergroup interactions (Wilson et al., 2003), or from the effective defences group-living insects can show, such as density-dependent immune defence (Barnes and Siva-Jothy, 2000; Reeson et al., 1998; Wilson et al., 2002), and enhanced behavioural or antimicrobial defences of groups against diseases (Hughes et al., 2002; Rosengaus et al., 1998; Traniello et al., 2002). There are, however, very few published surveys of the natural prevalence of social insect parasites. Such surveys in wasps (Foulliard and Morel, 1994; Rose et al., 1999), and termites (Milner et al., 1998; Ochiel et al., 1997), have on occasion revealed rich parasite communities to be associated with social insect colonies. The relative paucity of recorded parasites may therefore also be the result of a lack of investigation.

Species of the Deuteromycete fungal genera *Metarhizium* and *Beauveria* are virulent, generalist entomopathogens that are well established as infecting a wide variety of non-social insect hosts. There are also some records of them infecting social insects, but for these hosts the data is very limited (Schmid-Hempel, 1998). Both *Metarhizium* and *Beauveria* are 'obligate killers' (Ebert and Weisser, 1997), reproducing semelparously upon host death, and have consequently been much studied for their potential usefulness as biological control agents of many insect pests. They are, at least in temperate regions, principally associated with hosts in the soil environment, and thus soil-nesting social insects,

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such as most ants and termites, should be particularly prone to infection by them (Bequaert, 1921; Hölldobler and Wilson, 1990; Wheeler, 1910). Although these taxa are probably the most intensively studied entomopathogenic fungi, relatively little is known about their diversity in the environment. While they are known to be highly diverse across species or between widely separated populations (Berretta et al., 1998; Driver et al., 2000; Fegan et al., 1993; Fungaro et al., 1996; Pantou et al., 2003; Tigano-Milani et al., 1995), very few studies have looked at within population diversity. Data is particularly deficient in tropical areas, where the diversity of many organisms is highest.

Leaf-cutting ants (Hymenoptera: Formicidae: Attini) are dominant herbivores of the Neotropics and significant pests of many crops (Hölldobler and Wilson, 1990; Weber, 1972). Control methods are based predominantly upon the use of insecticide baits or the direct application of insecticide fogs to nests (Cherrett, 1986; Hughes and Goulson, 2002). Both methods are laborious, expensive, and leave a large chemical footprint in the environment. A number of studies have investigated the control of colonies using biological control agents, particularly the fungi *Beauveria* and *Metarhizium* (Diehl-Fleig et al., 1992b, 1993; Kermarrec et al., 1986; Silva and Diehl-Fleig, 1988; Silva et al., 1993). However, other than the occasional records of these fungi infecting individual leaf-cutting ants in the field (Alves and Sosa Gómez, 1983; Diehl-Fleig et al., 1992a; Humber, 1992; Jaccoud et al., 1999), nothing at all is known about how relevant these and other pathogens are to leaf-cutting ants in nature, either in terms of the frequency with which the contact them, the diversity of pathogen genotypes that they contact, or the actual impact such pathogens have upon colonies.

We set out to investigate the prevalence and diversity of fungal entomopathogens associated with and in the vicinity of leaf-cutting ant nests in an area of forest in the Neotropics. The aim was to establish what entomopathogenic fungi the ants were coming into contact with, how regular such contacts were likely to be, and how often infections appeared to occur.

2. Materials and methods

2.1. Sampling and isolation

Samples were collected from an area of approximately 2.25 km^2 in Gamboa, Panama during April 2001. The area could be divided into three habitats: (1) well established secondary forest with damp soil (close to a pond), (2) well established secondary forest with dry soil, and (3) young, scrubby secondary forest, graduating into grassland and gardens. The samples were collected from close to the nests of the four species of leaf-cutting ant that are commonly found in the area: *Atta cephalotes*, *Atta colombica*, *Acromyrmex octospinosus*, and *Acromyrmex echinatior*. Five nests were randomly selected for each species. For each nest, three sampling methods were adopted:

1. One hundred workers were collected from within the nest and fifty workers were collected as they returned to the nest carrying forage. In addition, in the following year samples of 20 dead workers and 20 alive, but unhealthy looking workers, were collected from the dump piles of each of five colonies of *A. colombica*. The living ants were maintained alive in isolation for five days with an ad libitum supply of water. All ants that died during this five day period, and all of those that were dead when collected, were surface sterilised (Lacey and Brooks, 1997) and stored in individual vials with moist cotton wool. The cadavers were checked daily for 5 days, and then weekly for four weeks for the external appearance of any fungal growth.
2. The vegetation within 5 m of the nest was searched for 1 h for insect cadavers.
3. Twenty soil cores of 5 cm depth by 3 cm diameter were collected randomly from within 5 m of the nest, excluding the nest mound. These cores were mixed and a 500 g sample stored at 5 °C. For each of the *A. colombica* nests, similar volumes of material were collected from the dump piles, which in this species are above ground and therefore readily accessible. Approximately 1 g of each soil or dump sample was weighed, oven-dried at 100 °C for 24 h, and reweighed to estimate the water content of the soil and dump samples. Each sample of soil or dump material was used to fill 10 pots, with each pot containing either 60 g of soil or 40 g of dump material. Five of these pots were used for live baiting for entomopathogenic fungi (Zimmerman, 1998) with *Galleria mellonella* (Lepidoptera: Pyralidae) and five with *Tenebrio molitor* (Coleoptera: Tenebrionidae), with six larvae being placed in each pot. The pots were kept moist at 20–22 °C and were revolved daily for four weeks. Cadavers were removed from the pots on a weekly basis, surface sterilised (Lacey and Brooks, 1997) if they had no external fungal growth, and maintained under humid conditions. Any fungal spores that were produced from the cadavers were plated on to both standard (Sabouraud dextrose agar) and selective (65 g Sabouraud dextrose agar, 1 ml 10% dodine (*N*-dodecylguanidine monoacetate), 1 ml 10% streptomycin sulphate, 1 ml 5% chloramphenicol, per litre of water) media plates. All soil and dump samples were also plated on to selective media plates. A 1 g amount of each sample was placed in a vial with 9 ml of 0.05% Triton-X solution, vortexed for three periods of 25 s, and serially diluted to give concentrations of 1×10^{0}, 1×10^{-1}, and 1×10^{-2}. One hundred microlitres of

each concentration of each sample was then plated on to each of three replicate plates of standard media, and three of selective media. Plates were stored at 24 °C and monitored weekly for four weeks for fungal sporulation.

2.2. Molecular methodology

The *Metarhizium anisopliae* var. *anisopliae* isolates obtained above were each isolated as monospores on to standard media plates. For DNA extraction, monospore isolates were grown in 15 ml liquid media (10 g peptone, 2 g yeast extract, 30 g sucrose per litre water) in 100 ml Ehrlenmayer flasks at 170 rpm at room temperature for two days. Mycelia were harvested by filtration on Whatman filter paper and freeze-dried. DNA was extracted from the freeze-dried mycelium following the protocol in Bulat et al. (1998), but using phenol/$CHCl_3$ (1:1) instead of $CHCl_3$/octanol (Jensen and Eilenberg, 2001). The DNA was amplified in Hybaid PCR Express Thermal Cyclers, using primers Ma-IGSspF and Ma-IGSspR (Pantou et al., 2003). These primers amplify a partial, 380 bp sequence of the IGS region that is highly polymorphic in *M. anisopliae* var. *anisopliae*, but which does not amplify in other species or varieties of *Metarhizium*. Reactions were carried out in volumes of 25 μl, consisting of 0.1 μl DNA, 2× reaction buffer, 0.25 mM dNTPs, 0.1 U *Taq* polymerase, and 0.1 μM of each of the primers. The temperature program had an initial denaturing step of 96 °C for 5 min, followed by a sequence of 35 cycles of 96 °C for 60 s, 58 °C for 60 s, and 72 °C for 60 s, and ended with a final elongation step of 71 °C for 10 min. PCR products were checked on agarose gels and purified using the Qiagen PCR purification kit. The purified products were then sequenced and the sequences edited using the Sequencher program. All other published *M. anisopliae* var. *anisopliae* sequences of the variable section of the IGS region were obtained from GenBank (Table 1). Sequences were aligned using Clustal-X and analysed with Paup 4.0 using parsimony and neighbour-joining methods with the general time-reversible model (Page and Holmes, 1998).

Table 1
Details of *M. anisopliae* var. *anisopliae* isolates whose sequences were obtained from GenBank

Isolate	Location	Host
ARSEF 439	Australia	*Teleogryllus commodus* (Orthoptera)
ARSEF 440	Australia	*Teleogryllus commodus* (Orthoptera)
ATHUM 2920	France	*Melolontha melolontha* (Coleoptera)
IMBST 9601	Austria	*Melolontha melolontha* (Coleoptera)
IMBST 9602	Austria	*Melolontha melolontha* (Coleoptera)
IMI 152222	India	*Myllocerus discolor* (Coleoptera)
IMI 298059	Papua New Guinea	*Scapanes australis* (Coleoptera)
IMI 298061	Papua New Guinea	*Brontispa longissima* (Coleoptera)
IMI 168777ii	Ethiopia	*Schistocerca gregaria* (Orthoptera)
ITALY 12	Italy	Pyralidae (Lepidoptera)
KVL130	Denmark	Noctuidae (Lepidoptera)
KVL275	Austria	*Cydia pomonella* (Lepidoptera)
KVL96-31	Denmark	Soil
KVL97	Denmark	Soil
KVL97125	Denmark	Soil
V38	United Kingdom	Forficulidae (Dermaptera)
V219	Unknown	Unknown
V242	Unknown	Unknown
V245	Finland	Soil
V248	Finland	Soil

3. Results

In total, 58 isolates of *Metarhizium* were obtained from soil samples, either by plating or live baiting. In addition, one isolate of *Metarhizium* was obtained from a dump pile of *A. colombica* by plating, and three isolates were obtained from unhealthy *A. colombica* workers collected from dump piles. *Metarhizium* appeared to be abundant in the habitat, with soil around 18 out of 20 nests proving positive for the fungus. Based on the six sites where *Metarhizium* was detected by plating, the density of *Metarhizium* in the soil ranged from 1×10^3 to 5×10^4 CFU g^{-1} soil. The density in the single dump sample positive for *Metarhizium* was an order of magnitude lower at 1×10^2 CFU g^{-1} dump material. All of the isolates of *Metarhizium* collected were identified as *M. anisopliae* var. *anisopliae* on the basis of their morphology. This identification was later confirmed by their amplification with the IGS primers specific to *M. anisopliae* var. *anisopliae*. No other varieties of *Metarhizium* were found. One soil sample was found by soil plating to contain the entomopathogen *Beauveria bassiana*, at an estimated density of 7.5×10^3 CFU g^{-1} soil. Of the isolation methods used, live baiting with *Tenebrio molitor* proved the most effective, detecting *M. anisopliae* var. *anisopliae* at 15/20 sites. Soil plating and live baiting with *G. mellonella* were less effective, isolating *M. anisopliae* var. *anisopliae* at 6/20 and 4/20 sites, respectively. Four of the five *A. colombica* dump piles were found by plating to contain *Aspergillus flavus*, which is a facultative pathogen of leaf-cutting ants (A.N.M. Bot unpublished; Hughes and Boomsma, 2003; Steinhaus and Marsh, 1967). None of the foragers or nest workers collected were found to be infected by entomopathogenic fungi and no sporulating leaf-cutting ant cadavers were found. However, six cadavers of another ant species, *Camponotus atriceps* (Formicidae: Formicinae), were found with *Cordyceps* sp. fruiting bodies on vegetation around the nests, as well as several cadavers of hemipteran bugs with unidentified fungal growth. None of the dead ants col-

lected from the dump piles showed signs of infection by an entomopathogenic fungus.

Forty-five of the *M. anisopliae* var. *anisopliae* isolates obtained were sequenced (41 of the soil samples, and all of the dump sample and dump ant isolates). These, and all 20 isolates of *M. anisopliae* var. *anisopliae* previously sequenced at the IGS region and available in GenBank (Table 1) were included in the analysis. Neighbour-joining and parsimony analyses produced identical trees. Sequences from GenBank formed three clades (A–C), as

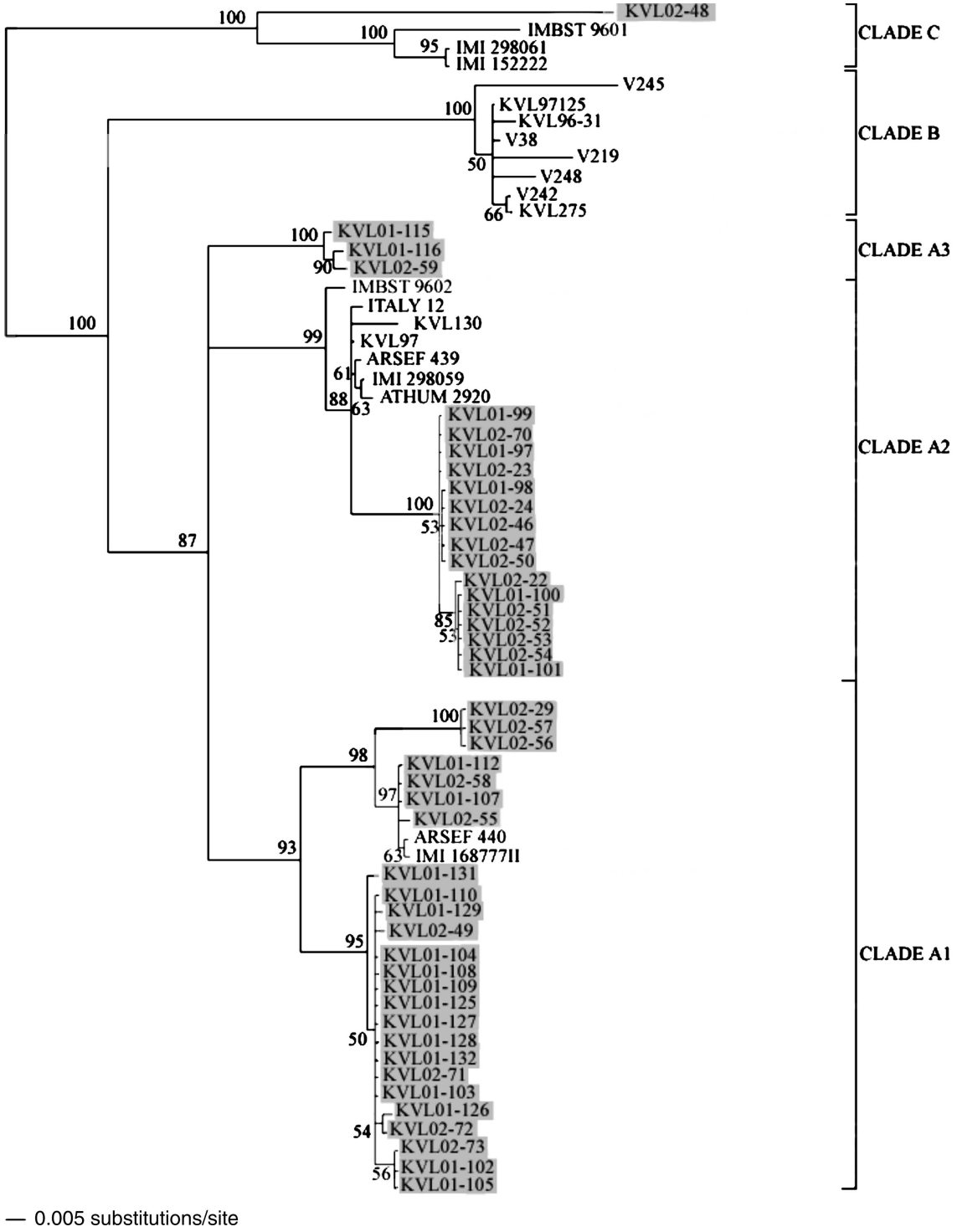

Fig. 1. Neighbour-joining tree of 45 Panamanian (shaded) and 20 other isolates of *M. anisopliae* var. *anisopliae* based on partial sequence of the IGS region. Bootstrap values are shown next to nodes and are based upon 1000 replicates. Distances were calculated using the general time-reversible method. The tree was rooted using isolates KVL02-48, IMBEST 9601, IMI 298061, and IMI 152222 as the outgroup.

found previously (Pantou et al., 2003) (Fig. 1). Forty-four of the forty-five Panamanian isolates fell within clade A, with the remaining isolate (KVL02-48) falling in clade C. The isolates within clade A were subdivided into three well supported clades (A1, A2, and A3), with Panamanian isolates being present in all three and non-Panamanian isolates in A1 and A2. The 25 Panamanian isolates in clade A1 came from the vicinity of 17 nests belonging to all four species of leaf-cutting ant, with the prevalence around any particular nest being low. In contrast, the 16 Panamanian isolates in clade A2 all came from the vicinity of *Atta* nests, with the prevalence around one *A. colombica* nest (Atco1) being very high. Clade A3 consisted of three isolates that all originated from around the same nest of *A. echinatior*.

There was no obvious relationship in the phylogeny with either the method by which the isolates were obtained (live baiting or soil plating) or the habitat they came from (Table 2). The water content of the soil was significantly higher at those sites located near the pond

Table 2
Details of *M. anisopliae* var. *anisopliae* isolates obtained from Panama field site and sequenced in this study

Isolate	Origin	Isolation method[a]	Ant sp. associate[b]	Nest	Habitat[c]	% Soil moisture	Morphology of spores (L/W)
KVL01-97	Soil	T	At co	1	F	28.4	2.52
KVL01-98	Soil	T	At co	1	F	28.4	2.46
KVL01-99	Soil	T	At co	1	F	28.4	2.60
KVL01-100	Soil	T	At co	1	F	28.4	2.64
KVL01-101	Soil	T	At co	2	F	28.8	2.91
KVL01-102	Soil	G	At co	5	W	24.0	2.87
KVL01-103	Soil	T	At ce	3	F	24.5	2.52
KVL01-104	Soil	T	At ce	3	F	24.5	2.50
KVL01-105	Soil	T	At ce	4	F	27.4	2.50
KVL01-107	Soil	T	Ac oc	1	F	27.0	2.67
KVL01-108	Soil	G	Ac oc	2	F	27.0	2.92
KVL01-109	Soil	T	Ac oc	4	F	28.6	2.92
KVL01-110	Soil	T	Ac ec	1	F	22.5	2.58
KVL01-112	Soil	T	Ac ec	3	S	27.8	2.58
KVL01-115	Soil	T	Ac ec	5	S	28.3	2.60
KVL01-116	Soil	T	Ac ec	5	S	28.3	2.68
KVL01-125	Soil	T	Ac ec	4	S	25.0	2.82
KVL01-126	Soil	G	Ac oc	4	F	28.6	2.70
KVL01-127	Soil	T	Ac oc	3	F	29.9	2.60
KVL01-128	Soil	T	Ac ec	4	S	25.0	2.83
KVL01-129	Soil	T	At co	2	F	28.8	2.82
KVL01-131	Soil	T	At co	3	W	28.6	2.91
KVL01-132	Soil	T	Ac oc	1	F	27.0	2.92
KVL02-22	Soil	T	At co	1	F	28.4	2.44
KVL02-23	Soil	T	At co	1	F	28.4	2.10
KVL02-24	Soil	G	At co	1	F	28.4	2.71
KVL02-29	Soil	T	At ce	2	F	25.5	2.76
KVL02-46	Soil	P	At ce	2	F	25.5	2.52
KVL02-47	Soil	P	At ce	2	F	25.5	2.65
KVL02-48	Soil	P	At ce	4	F	27.4	3.04
KVL02-49	Soil	P	At ce	5	F	31.5	2.82
KVL02-50	Soil	P	At co	1	F	28.4	2.52
KVL02-51	Soil	P	At co	1	F	28.4	2.21
KVL02-52	Soil	P	At co	1	F	28.4	2.56
KVL02-53	Soil	P	At co	1	F	28.4	2.56
KVL02-54	Soil	P	At co	1	F	28.4	2.58
KVL02-55	Soil	T	At co	4	W	38.5	2.64
KVL02-56	Dump	P	At co	4	W	54.2	2.85
KVL02-57	Soil	T	Ac ec	2	S	27.8	2.77
KVL02-58	Soil	T	Ac ec	3	S	29.7	2.54
KVL02-59	Soil	P	Ac ec	5	S	28.3	2.67
KVL02-70	Dump	Ant	At co	6	W	57.4	3.04
KVL02-71	Dump	Ant	At co	9	F	54.2	2.85
KVL02-72	Dump	Ant	At co	9	F	54.2	2.83
KVL02-73	Soil	T	At ce	4	F	27.4	2.75

[a] P, plating; T, baiting with *T. molitor*; G, baiting with *G. mellonella*; Ant, isolated from *A. colombica* worker.
[b] Ac ec, *Acromyrmex echinatior*; Ac oc, *Acromyrmex octospinosus*; At ce, *Atta cephalotes*; At co, *Atta colombica*.
[c] W, wet secondary forest, near pond; F, dry secondary forest; S, scrubby forest graduating into grassland.

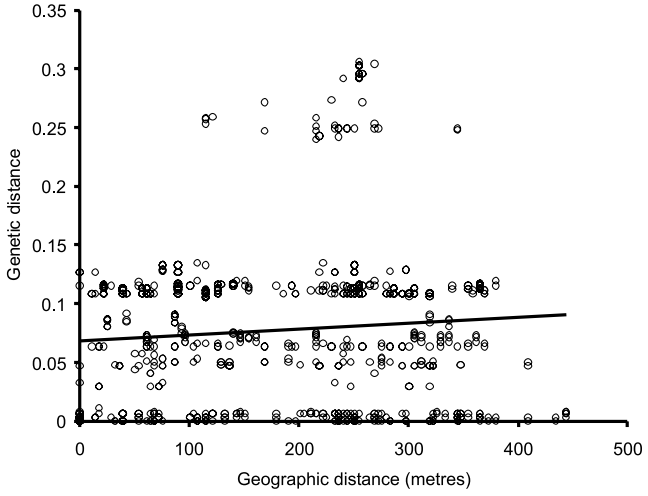

Fig. 2. Genetic and geographical distance between 45 isolates of *M. anisopliae* var. *anisopliae* collected at field site in Panama. Points represent each pair of interactions between different isolates. Genetic distances were calculated using the general time-reversible method. Line of best fit is $y = 0.00005x + 0.0686$.

(40.5% ± 6.68) than those located elsewhere (dry secondary forest: 29.1 ± 1.80%; young secondary forest/grassland: 27.7 ± 0.99%) ($F_{2,21} = 3.70$, $P = 0.042$). It was also significantly higher near nests of *A. colombica* (which were more often located in the area of forest near the pond) than nests of the other species ($F_{3,20} = 76.5$, $P < 0.001$), and was far higher in dump material (53.6 ± 2.30%) than in soil (27.8 ± 0.74%) ($F_{4,20} = 47.0$, $P < 0.001$). However, the four clades of Panamanian isolates (A1, A2, A3, and C) did not differ in the water content of the soil from which they were isolated ($F_{2,19} = 0.688$, $P = 0.515$). Neither did they differ in the dimensions of their spores ($F_{2,19} = 0.433$, $P = 0.655$). There was also no significant correlation between the genetic and geographical distance separating the Panamanian isolates (Mantel test with 1×10^6 permutations: $r = 0.095$, $P = 0.120$) (Fig. 2). The occurrence of four genetically distinct clades was quite obvious within this dataset (Fig. 2). Although the overall effect of geography was therefore only slight, there was a strong tendency for isolates collected from around the same nest to be closely related. Isolates collected around the same nest were separated by a genetic distance of 0.007 ± 0.003 (mean ± SE), whereas isolates collected from around different nests were separated by a distance of 0.083 ± 0.002 ($t = 10.9$, df = 988, $P < 0.001$).

4. Discussion

Leaf-cutting ants at the site in Panama would appear to be regularly exposed to *M. anisopliae* var. *anisopliae*, with the majority of colonies sampled having *M. anisopliae* var. *anisopliae* present in at least the upper 5 cm soil layer in their vicinity. Leaf-cutting ant colonies can be extremely large, with those of *Atta* spp. being as much as 20 m in diameter and 7 m deep (Jonkman, 1976). A very large volume of soil needs to be worked in order to create and maintain such nests, and so contact between leaf-cutting ant workers and soil will be common. *A. flavus* was commonly found in the dump piles of *A. colombica*, and is also common in the dump piles of *A. echinatior* and *Ac. octospinosus* (Hughes unpublished). This fungus has previously been noted as infecting leaf-cutting ants (A.N.M. Bot unpublished; Hughes and Boomsma, 2003; Steinhaus and Marsh, 1967), as well as *Solenopsis* fire ants, wasps, bees, and termites (Beal and Kais, 1962; Glare et al., 1996; Harris et al., 2000; Lund, 1965; Pereira and Stimac, 1997; Schmid-Hempel, 1998). It appears to be a facultative pathogen that is most probably soil borne, and thus which leaf-cutting ants will again come into regular contact with. Both *B. bassiana* and *Cordyceps* sp., with the latter possibly being the teleomorph of an entomopathogenic Deuteromycete (Liu et al., 2002), were also found and are additional pathogens that leaf-cutting ants will be less commonly exposed to.

In the soil sampled, *M. anisopliae* var. *anisopliae* appeared to dominate the entompathogenic mycoflora. No other species or varieties of *Metarhizium* were found, and *B. bassiana* was isolated from only one soil sample. *M. anisopliae* var. *anisopliae* was also noticeable in its abundance with 18/20 sites sampled being positive for it. This compares with the lower abundances of entomopathogenic fungi often recorded in agricultural habitats, where between <5 and 30% of sites were positive (Ali-Shtayeh et al., 2002; Chandler et al., 1997; Klingen et al., 2002; Rath et al., 1992; Vänninen, 1996), although such data should be seen in light of the isolation method used (Klingen et al., 2002). Near termite nests, in both a study very similar to the present one (Milner et al., 1998) and one in which isolates were obtained by baiting with termites (Sun et al., 2003), only about 16% of samples were positive.

The genetic diversity of *M. anisopliae* var. *anisopliae* isolates within the small geographical area sampled was surprisingly high, with 14 distinct types being detected. All of these apart from one came from clade 'A' of the three *M. anisopliae* var. *anisopliae* clades previously identified by Pantou et al. (2003). Those in clade A, were themselves subdivided into three well-supported groups. Two of these contained isolates from elsewhere in the world, while the third clade (A3) contained only isolates from the area of Panama sampled. Previously, *Metarhizium* genotypes have often been found to be associated with either host species (Bridge et al., 1997; Fegan et al., 1993; St. Leger et al., 1992; Tigano-Milani et al., 1995) or habitat (Bidochka et al., 2001, 2002; Vänninen, 1996). There was no association with habitat amongst the isolates described in this study, and there was only

limited evidence of particular genotypes being associated with particular host ant species. The isolation of clade A3 from only a single *A. echinatior* nest may simply reflect an underdispersed distribution, but the association of Panamanian subclade of A2 with *Atta* was better supported, with it originating from four, widely distributed nests.

In spite of the high prevalence of entomopathogenic fungi around their colonies, very few ants were actually infected. This was even when workers were maintained in isolation, and thus deprived of the group level defences that play such a key role in protecting them, and other social insects against diseases (Hughes et al., 2002; Jaccoud et al., 1999; Kermarrec et al., 1986; Rosengaus et al., 1998; Schmid-Hempel, 1998; Traniello et al., 2002). In addition to their group defences though, leaf-cutting ants are also protected by individual defences, such as selfgrooming and antibiotic excretions, that are known to be effective at protecting against parasites such as *Metarhizium* (Hughes et al., 2002; Poulsen et al., 2002). It would appear that these individual defences, together with the immune system, are normally able to protect leaf-cutting ants against entomopathogenic fungi, although, as the few infected ants found on the dump piles shows, the defences are not always effective.

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