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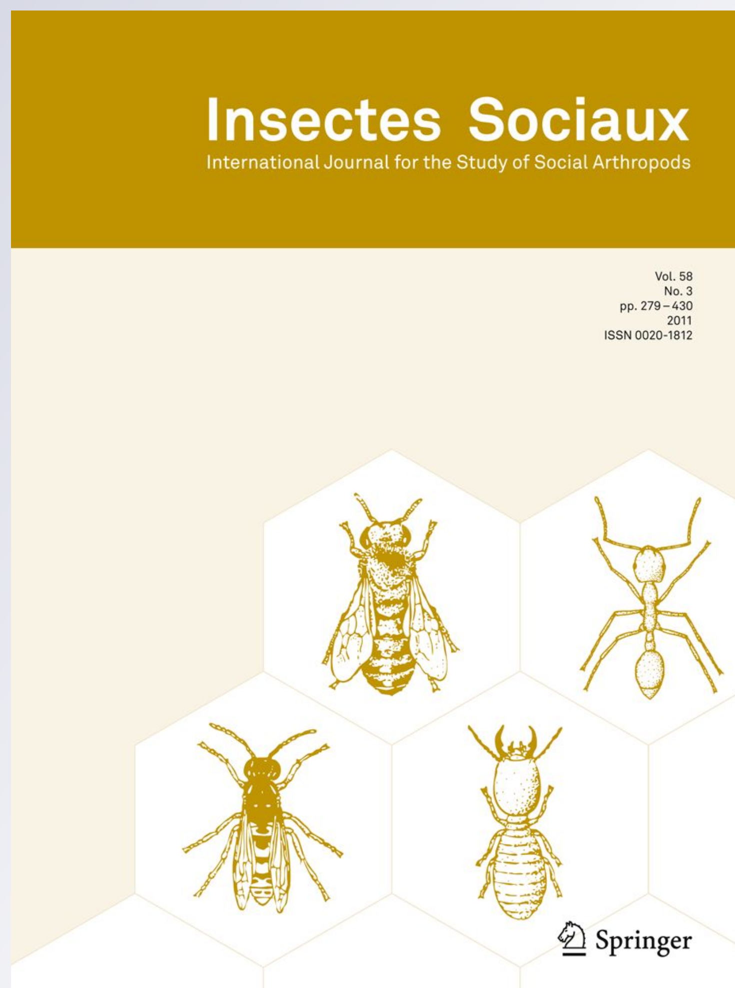

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Insectes Sociaux

RESEARCH ARTICLE

Diversity, prevalence and virulence of fungal entomopathogens in colonies of the ant *Formica selysi*

A. Reber · M. Chapuisat

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Abstract The richness of the parasitic community associated with social insect colonies has rarely been investigated. Moreover, understanding how hosts and pathogens interact in nature is important to interpret results from laboratory experiments. Here, we assessed the diversity, prevalence and virulence of fungal entomopathogens present around and within colonies of the ant *Formica selysi*. We detected eight fungal species known to be entomopathogenic in soil sampled from the habitat of ants. Six of these entomopathogens were found in active nests, abandoned nests, and corpses from dump piles or live ants. A systematic search for the presence of three generalist fungal entomopathogens in ant colonies revealed a large variation in their prevalence. The most common of the three pathogens, *Paecilomyces lilacinus,* was detected in 44% of the colonies. *Beauveria bassiana* occurred in 17% of the colonies, often in association with *P. lilacinus*, whereas we did not detect *Metarhizium brunneum* (formerly *M. anisopliae*) in active colonies. The three fungal species caused significant mortality to experimentally challenged ants, but varied in their degree of virulence. There was a high level of genetic diversity within *B. bassiana* isolates, which delineated three genetic strains that also differed significantly in their virulence. Overall, our study indicates that the ants encounter a diversity of fungal entomopathogens in their natural habitat. Moreover, some generalist pathogens vary greatly in their virulence and prevalence in ant colonies, which calls for further studies on the specificity of the interactions between the ant hosts and their fungal pathogens.

Keywords Host–parasite · Soil fungi · Parasite diversity · Ant pathogens · Social insects · *Formica selysi*

Introduction

Social insects might be particularly susceptible to parasites, because they live in dense groups of related individuals (Schmid-Hempel, 1998). However, few studies have described parasitic diversity in wild populations of social insects (Schmid-Hempel, 1998; Hughes et al., 2004). Data on parasite richness and host–parasite relationships in natural populations are important to understand how anti-parasitic defences evolved and to interpret immunological or behavioural results from laboratory experiments.

Deuteromycete fungi belonging to the genera *Metarhizium* and *Beauveria* infect a wide variety of non-social insect hosts (Meyling and Eilenberg, 2007). These soil fungi are obligate-killers, which grow inside their hosts and kill them to produce a large number of transmission propagules (Ebert and Weisser, 1997). The possibility to experimentally infect hosts and the good traceability of their asexual spores (= conidia) make them ideal model systems to monitor the impact and spread of infection (e.g. Jaccoud et al., 1999). As a result, species belonging to the *Metarhizium anisopliae* complex have been widely used in experimental studies of disease resistance in social insect colonies (e.g. Rosengaus et al., 1998; Hughes et al., 2002; Ugelvig and Cremer, 2007; Reber et al., 2008, 2011).

Most studies investigating the natural occurrence and virulence of fungal parasites have focused on their interaction with insect pest populations for biocontrol purposes (e.g. Hegedus and Khachatourians, 1996a; Pirali-Kheirabadi et al., 2007; Santos et al., 2007). In contrast, only a couple of surveys have described the fungal community that occurs in

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the habitat of social insects (Milner et al., 1998; Hughes et al., 2004). Moreover, very little is known about the prevalence and diversity of fungal pathogens actually infecting social insects in natural conditions (Briano et al., 1995; Pereira, 2004; Rodrigues et al., 2010; Evans et al., 2011).

The inter- and intra-specific diversity of pathogens may both have important implications for the host species. We searched for entomopathogenic fungal species infecting ant colonies and further investigated the genetic diversity occurring within one of them, *Beauveria bassiana.* This fungus principally reproduces asexually by mitosporic spores (conidia) and has long been presumed to be entirely clonal. However, the recent molecular studies have detected that *B. bassiana* may be facultatively sexual, with heterothallic outcrossing and predominance of clonal reproduction due to strong skew in mating-type frequencies (Meyling et al., 2009). The genetic diversity, mode of reproduction and distribution of *B. bassiana* strains has not been investigated so far in social insect populations.

We assessed the diversity, prevalence and virulence of entomopathogenic fungi in the habitat and within colonies of *Formica selysi*. This species of ants nests in the soil and forages above the ground as well as in the vegetation, where it collects various arthropod prey (Keller and Zettel, 2001). Hence, *F. selysi* is likely to be exposed to fungal pathogens present in the soil or in preys. We first sampled the diversity of fungal entomopathogens present in the soil inhabited by the ants, using fungal culture or *Tenebrio* baiting of soil samples, followed by direct sequencing of the isolated fungi. We then examined whether the ants were actually infected by the fungal parasites present around their nests and whether the frequency of infection differed among fungal species. For this, we combined systematic sampling and molecular typing to detect infection by three generalist fungal pathogens within ant colonies: *Metarhizium brunneum* (previously called *M. anisopliae,* now distinguished as a proper sibling species, Bischoff et al., 2009), *B. bassiana* and *Paecilomyces lilacinus*. We further characterized the genetic diversity within isolates of *B. bassiana* with microsatellites. Finally, we compared the virulence of the *M. brunneum, P. lilacinus* and *B. bassiana* strains present in our field population. These results will shed light on the interactions between ant hosts and their fungal pathogens in natural conditions.

Methods

Study site

Our study site is located in the alluvial plain of the river Rhône, between Sierre and Susten in the central Valais, Switzerland. Nests of *Formica selysi* occur at high density (Chapuisat et al., 2004; Rosset and Chapuisat, 2007). The samples were collected from an area approximately 1.5 km long and 500 m wide. The habitat consists in a mosaic of pine forest, steppe areas dominated by the grass *Stipa pennata* and floodplains of bare sand and gravel.

Identification of entomopathogenic fungi in the soil

In order to detect and identify the presence of entomopathogenic fungi in soil samples, we used fungal culture and *Tenebrio* baiting methods, followed by molecular typing. In September 2006, we collected 41 soil samples in the diverse types of habitat frequented by *F. selysi* (Table 1). Out of these 41 samples, 21 came from areas surrounding nests, nine from abandoned nests and 11 from active nests. We collected soil samples with a flame-sterilized cylindrical soil corer (inner diameter 2 cm, length 18 cm), mixing two soil cores 0.2 m apart for each sample. The soil samples were stored at 4°C for less than a week.

For the fungal culture on selective artificial medium, we passed each soil sample through a sterile 2-mm sieve, diluted 1 g of soil in 5 ml of sterile water containing 0.05% of Tween 20 as surfactant, and spread 200 µl of this soil dilution on cultivation plates. The cultivation plates contained a selective medium supplemented with antibiotics (streptomycine and tetracycline) and antimycotics (cycloheximidine and dodine; for medium composition and preparation, see Keller et al., 2003). This medium, originally designed for the selective isolation of *Beauveria brongnartii*, has been shown to be appropriate for the isolation of multiple soil entomopathogenic fungi (Keller et al., 2003). For each of the 41 soil samples, we made two replicate plates, which were kept at 25°C for 5–7 days to permit the development of fungal colonies.

For the *Tenebrio* baiting, we buried ten young larvae of yellow mealworm beetles (*Tenebrio molitor*) in 50 g of soil from each sample. We covered the soil with wet cotton wool, in small glass pots that we revolved daily to keep the larvae moving in the soil. We checked the mortality of the mealworm beetles on a weekly basis for 1 month. To detect the presence of internal fungal pathogens, the cadavers were surface sterilized, placed on the selective medium described above and kept at 25°C for 5 days to permit the emergence of fungal spores (Lacey, 1997). We verified that the *T. molitor* larvae were not infected by fungal pathogens before being placed in the soil samples: none of 10 cadavers of *T. molitor* collected from the stock and treated as described above produced fungal spores.

We classified the fungal isolates into morphotypes based on morphological similarities. To identify the fungal species, we sequenced the ITS1-5.8S-ITS2 nuclear ribosomal cistron from one to three isolates of each morphotype. When we detected an entomopathogen, we further sequenced the

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Table 1 Fungi isolated from soil sampled in the habitat of *F. selysi* in the alluvial plain of the river Rhône, Valais, Switzerland

Species (top match in GenBank)	Isolation method	Habitat type	Prop. positive samples ($n = 41$) (%)	Entomopathogen	References
Aspergillus flavus	T	St	14.6	Yes	(Drummond and Pinnock, 1990)
Aspergillus parasiticus	T	AbN, AcN, B, FE	9.7	Yes	(Drummond and Pinnock, 1990)
Auxarthron conjugatum	P	St	2.4	–	
Beauveria bassiana	P, T	St, RB	12.2	Yes	(Meyling and Eilenberg, 2007)
Penicillium sizovae	T	AbN	17.1	–	
Gloeotinia temulenta/Verticillium leptobactrum	P	St	2.4	–	
Aphanocladium aranearum	T	B	2.4	–	
Lecanicillium psalliotae	T	B	2.4	Yes	(Pirali-Kheirabadi et al., 2007)
Metarhizium brunneum (formerly *M. anisopliae*)	T	AbN, St	4.9	Yes	(Meyling and Eilenberg, 2007; Bischoff et al., 2009)
Mortierella alpina	P, T	PF	4.9	–	
Mortierella verticillata	P	AcN	2.4	–	
Mucor fragilis	P	B	2.4	–	
Mucor hiemalis	P, T	AbN, St	12.2	Yes	(Konstantopoulou et al., 2006)
Neosartorya aureola/Neosartorya udagawae	T	AcN	2.4	–	
Neosartorya sp.	P	St	2.4	–	
Paecilomyces lilacinus	P, T	AbN, FE, St	22	Yes	(Rodrigues et al., 2010)
Penicillium skrjabinii	T	AbN	2.4	Yes	(Humber et al., 2009)
Petromyces alliaceus	P, T	AbN, AcN, St	26.8	–	
Fungus sp.	P	AcN, PF	9.8	–	

The sequence identity at the ITS1-5.8S-ITS2 nuclear ribosomal cistron was comprised between 98 and 100%

P plating, *T* baiting with *T. molitor*, *AbN* abandoned nest of *F. selysi* (9 samples), *AcN* active nest of *F. selysi* (11 samples), *B* bank (4 samples), *FE* forest edge (2 samples), *PF* pine forest (3 samples), *St* steppe (10 samples), *RB* river bed (sand and gravel, 2 samples)

other isolates of the same morphotype. We extracted the fungal DNA with a phenol–chloroform protocol, PCR-amplified and sequenced the ITS1-5.8S-ITS2 nuclear ribosomal cistron as described in Pantou et al. (2003). We compared the sequences to the ones deposited in the National Center for Biotechnology Information using the Blast 1.4 10MP program (http://www.ncbi.nlm.nih.gov/). In addition, when the ITS1-5.8S-ITS2 sequence matched to the *Metarhizium anisopliae* species complex, we further sequenced the *elongation factor-1 alpha* gene region, which permitted us to identify the species as being *M. brunneum* (Bischoff et al., 2009).

Prevalence of three generalist fungal pathogens in colonies of *F. selysi*

We surveyed the prevalence of three generalist fungal entomopathogens in field colonies of *F. selysi*. We combined systematic sampling and molecular typing to detect infection by *M. brunneum*, *B. bassiana* and *P. lilacinus* (Tiganomilani et al., 1995; Fiedler and Sosnowska, 2007). We sampled ants that died in captivity within 5 days after having been collected from the field, as well as corpses from dump piles. We surface sterilized all corpses and placed them on a selective medium to detect the production of spores from internal fungal pathogens. When the corpses produced fungal colonies on the selective medium, we screened the fungal colonies by PCR amplifications with specific primers.

In August, 2007, we collected a large sample of live ants from each of 81 field colonies. *Formica selysi* has some division of labour among workers (Schwander et al., 2005). We selectively collected foragers, which go out of the nests and are therefore more likely to encounter fungal parasites. For this, we placed an open plastic box (13.5 cm long × 15 cm wide × 5 cm high) on top of each nest and waited until approximately 50 ants had walked into the box. The boxes were lined with Fluon GP1 (Whitford Plastics, Diez, Germany) to prevent ants from escaping. The ants were kept in the laboratory at 25°C under a 12 h day/night cycle, with ad libitum access to water and a protein-rich jelly food (Meunier and Chapuisat, 2009). We checked the mortality of ants daily over five days and collected the corpses of freshly dead ants for further analysis. This procedure and time-frame ensured that we detected ants that were already infected in the field. In addition, we collected corpses of ants

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from dump piles located near the entrance of each of the 81 field colonies. These dump piles result from the action of workers, which carry corpses and wastes to well-defined location out of the nest.

To detect actual infection and not the mere presence of fungal spores, we surface-sterilized all corpses from ants that died shortly after collection, as well as all corpses from dump piles, and placed them on selective medium at 25°C for five days to monitor the emergence and sporulation of internal pathogenic fungi (Lacey, 1997). The corpses that produced fungal conidia were stored at 4°C for subsequent PCR-typing of the three target fungal pathogens.

When corpses produced fungal colonies, we identified the three target fungal species by PCR amplifications using specific primers. The PCR protocols were adapted from Pantou et al. (2003), Atkins et al. (2005) and Hegedus and Khachatourians (1996a), (1996b). We used a phenol–chloroform method to extract the DNA from every corpse that produced fungal conidia. We detected *M. brunneum* by amplifying part of the IGS region (380 bp) using the primers Ma-IGSspF (CTACC(C/T)GGGAGCCCAGG CAAG) and Ma-IGSspR (AAGCAGCCTACCCTAAAGC; Pantou et al., 2003); *P. lilacinus* by amplifying the ITS gene (130 bp) with the primers PaeF (CTCAGTTGCCTCGGCG GGAA) and PaeR (GTGCAACTCAGAGAAGAAATTC CG; Atkins et al., 2005); and *B. bassiana* by amplifying the mtDNA EH1 gene (494 bp) with the primers P1 (AAGC TTCGACATGGTCTG) and P5 (AGGAGAGAGCTCGA CGGTCA; Hegedus and Khachatourians, 1996b).

The PCR reactions contained 5–25 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM of each deoxyribonucleotide, 2.5 U of Qiagen Taq polymerase, 1× Qiagen PCR Buffer (with 1.5 mM $MgCl_2$) and 1× Qiagen Q solution in a final volume of 50 µl. The PCR reactions for *B. bassiana* and *M. brunneum* had an additional 0.5 mM of $MgCl_2$. The PCRs were cycled 35 times (*M. brunneum* and *P. lilacinus*) or 25 times (*B. bassiana*) at 95°C for 30 s, annealing temperature (52, 55 and 60°C for *B. bassiana*, *M. brunneum* and *P. lilacinus*, respectively) for 30 s and 72°C for 45 s, with a final elongation at 72°C for 7 min. Aliquots of the amplification products (5 µl) were run on 1.5% (*M. brunneum* and *B. bassiana*) or 2% (*P. lilacinus*) agarose gels, stained with ethidium bromide and visualized under UV light.

In two cases, the amplification products obtained with *B. bassiana* primers had unexpected sizes. The sequencing of these products revealed that the primers had cross amplified the non-entomopathogenic fungus *Aspergillus lentulus.* These two cases were excluded from further analysis. The identification of the three target fungal pathogens was further confirmed by their coloration: *P. lilacinus* cultivated on nutritive medium had a pink stain, whereas *B. bassiana* and *M. brunneum* conidia were hyaline and green, respectively.

Genetic diversity within *B. bassiana*

We sequenced the ITS1-5.8S-ITS2 nuclear ribosomal cistron and the mitochondrial EH1 gene of 17 isolates of *B. bassiana* obtained from ant corpses and two isolates from soil samples. We aligned and compared the sequences with the software Sequence Navigator (Parker, 1997). The sequence variation at the two genes differentiated three well-separated genetic strains (see "Results").

We determined the genotypes of the 19 isolates of *B. bassiana* at six microsatellite loci (Ba01, Ba02, Ba03, Ba05, Ba12, Ba13; Rehner and Buckley, 2003; McGuire et al., 2005). The DNA amplification was adapted from McGuire et al. (2005), starting with 10 cycles of a touchdown PCR with annealing temperature decreasing from 50 to 40°C, followed by 36 cycles with annealing temperature of 50°C. The genetic differentiation within and between strains of *B. bassiana* was investigated with a neighbour-joining tree based on the multi-loci microsatellite genotypes using the online software pubMLST (http://pubmlst.org, Maiden et al., 1998).

Virulence of *M. brunneum*, *P. lilacinus* and *B. bassiana* strains

We first examined the impact of *M. brunneum, P. lilacinus* and *B. bassiana* (strain S2) on ant survival, and further compared the effect of each of the three genetic strains of *B. bassiana.* We cultured each fungal strain on a nutritive medium (Malt extract agar) at 25°C for 5–7 days to obtain conidia that we harvested into sterile water with 0.05% Tween 20. We measured the concentration of conidia with a haemocytometer (Neubauer improved counting chamber) and adjusted it to 2×10^7 conidia/ml. The experiment involved a total of 360 ants. For each of the three fungal species and the control, we challenged 20 workers sampled equally from 10 field colonies. For each of the three genetically different strains of *B. bassiana* and the control, we challenged 70 workers sampled equally from 10 field colonies. We deposited one microliter of conidia solution or control solution (sterile 0.05% Tween 20) on the thorax of each treated or control ant, respectively. We chose this dose and mode of application because they caused significant mortality in a previous study wherein we exposed *F. selysi* to the same strain of *Metarhizium* (Reber et al., 2011). After the exposure to the fungus, we placed each tested ant individually in a small Petri dish (35 mm diameter, 15 mm height) containing jelly food and a filter paper (2 × 2 cm) humidified daily. We monitored their survival daily for up to 15 days. In other experiments, we found that *F. selysi* workers exposed to fungal conidia as described above and surface-sterilized after death sporulated in 83–92% of the cases for *B. bassiana* (Reber and Chapuisat, unpubl. data)

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and in 90% of the cases for *M. brunneum* (Reber et al., 2011), which confirms that these two fungi cause internal infection.

We analysed the survival of ants with parametric survival models, using the function *survreg* of the program R version 2.8.1 (R Development Core Team, 2006). To assess the virulence of the three fungal species, we evaluated the effect of the treatment (exposure to each fungal species or control) with a likelihood Chi-square ratio test. We included the colony of origin as a random factor, using the frailty argument for the *survreg* function (Therneu et al., 2003). To analyse the virulence of the three genetic strains of *B. bassiana*, we evaluated the effects of two fixed factors, the treatment (exposure to each *B. bassiana* strain or control) and colony of origin, as well as their interaction, with likelihood Chi-square ratio tests. We sequentially removed non-significant terms. We selected a Weibull distribution of error, which produced the minimum error deviance.

Results

Identification of entomopathogenic fungi in the soil

The fungal colonies isolated from soil samples were classified into 38 morphotypes based on differences in their morphology such as shape, texture and colour. The sequence of the nuclear ribosomal cistron ITS1-5.8S-ITS2 permitted us to identify 19 fungal species (Table 1). Among these 19 species, 8 were entomopathogenic according to the literature (see Table 1 and references herein). Each of these eight fungal species was detected in some of the *Tenebrio* baiting, which corroborates their entomopathogenic properties.

We found entomopathogenic fungi in four of the five types of habitat sampled (steppe, forest edge, river bank, and river bed but not pine forest). We detected four entomopathogenic fungal species in the soil of abandoned nests of *F. selysi*. One of these species was also found in the soil of an active nest. The overall frequency of detection in soil samples varied among fungal species, ranging from 2.4 to 22% for entomopathogenic species (Table 1).

Prevalence of three generalist fungal pathogens in colonies of *F. selysi*

We obtained a snapshot estimate of the prevalence of *M. brunneum*, *B. bassiana* and *P. lilacinus* by systematically typing all ant corpses that showed signs of internal fungal infection. The PCR-typing of each of the three fungal pathogens revealed pronounced differences in their prevalence within the 81 field colonies surveyed. In total, 152 corpses of ants that died shortly after collection (1.9 per colony, on average) and 169 corpses from dump piles (2.1 per colony, on average) produced fungal conidia after surface sterilization. These corpses were subjected to PCR typing. *Paecilomyces lilacinus* was detected in a total of 36 colonies, both in live ants that died shortly after field sampling (14 individuals from 13 colonies) and in corpses originating from dump piles (61 individuals from 25 colonies). *Beauveria bassiana* was detected in 14 colonies, but only in live ants that died shortly after field sampling (17 individuals). It was not found in corpses from dump piles. An intriguing finding was that six of the 14 colonies in which we found *B. bassiana* also contained *P. lilacinus* in corpses originating from ants collected alive, which reveals a non-random association of the two pathogens in field colonies (binomial test, $p = 0.018$). We did not detect *M. brunneum* in active colonies, neither in cadavers from dump pile nor in corpses originating from ants collected alive. The colonies in which we detected fungal infections were distributed over the entire study area, suggesting that there was no marked spatial segregation of the pathogens.

Genetic diversity within *B. bassiana*

The ITS1-5.8S-ITS2 and EH1 genes were polymorphic in *B. bassiana.* Both genes had three well-differentiated haplotypes delineating three genetic strains. The sequence divergence between the strains S1 and S2 was 3.5 and 13.6% for the ITS1-5.8S-ITS2 and EH1 genes, respectively. S3 differed from S1 by 11 and 0.4%, and from S2 by 8 and 14% for ITS1-5.8S-ITS2 and EH1, respectively. There was no sequence polymorphism within strains and complete linkage between alleles at the two genes within each strain.

The most frequent strain (S1) was found in 15 isolates that came from ten ant colonies and two soil samples. The second strain (S2) was found in three colonies and the third strain (S3) in a single colony. The three strains occurred in the same areas and showed no marked spatial clustering. In two cases the S1 and S2 strains were found in neighbour colonies, and the colony containing S3 was close to a colony containing S2.

The genotyping of six microsatellite loci revealed polymorphism within and between strains of *B. bassiana*. The overall microsatellite diversity was high, as we detected nine different haploid genotypes (= haplotypes) out of 19 isolates (six haplotypes in strain S1, two in S2 and one in S3). The microsatellite diversity confirmed the genetic differentiation between the strains (Fig. 1). Strain S1 had no allele in common with strains S2 and S3, except one allele at one marker found in one ant, whereas strains S2 and S3 differed at three loci. We found one ant colony that contained two different *B. bassiana* haplotypes. Conversely, colonies separated by up to 581 m contained identical *B. bassiana* haplotypes.

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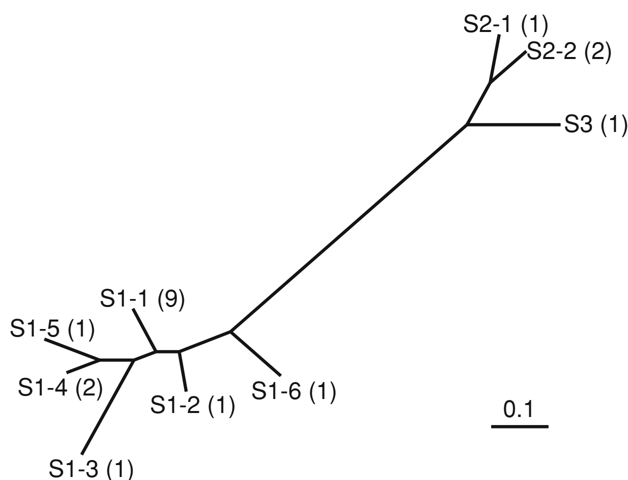

Fig. 1 Unrooted neighbour-joining tree based on the multi-loci microsatellite genotypes of *Beauveria bassiana*. Each haplotype is designated by the number of the strain S1, S2 or S3 on the left and a distinctive number on the right. The number of isolates per haplotype is given in *parentheses*

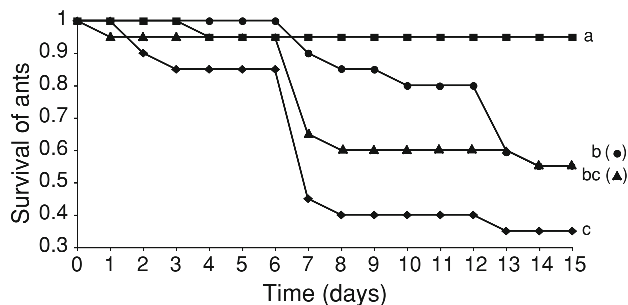

Fig. 2 Proportion of ants surviving after being challenged with *Beauveria bassiana* strain S2 (*triangles*), *Paecilomyces lilacinus* (*circles*), *Metarhizium brunneum* (*diamonds*) or a control solution (*squares*). *Different letters* indicate treatments that differed significantly from one another

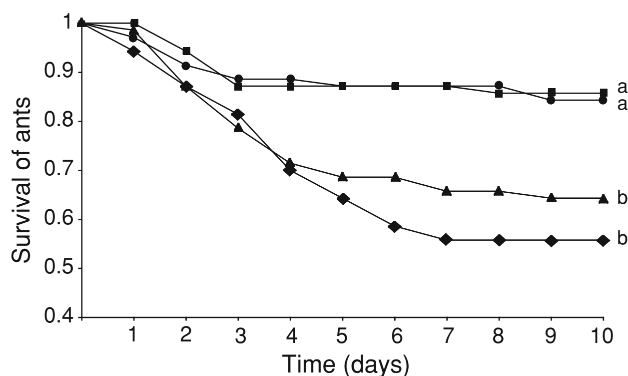

Fig. 3 Proportion of ants surviving after being challenged with *Beauveria bassiana* strain S1 (*circles*), S2 (*diamonds*), S3 (*triangles*), or a control solution (*squares*). *Different letters* indicate treatments that differed significantly from one another

Virulence of *M. brunneum*, *P. lilacinus* and *B. bassiana* strains

The survival of ants exposed to *M. brunneum*, *P. lilacinus*, *B. bassiana* (strain S2) and to a control solution differed significantly (Fig. 2, deviance = 29.6, $df = 4.4$, $p < 0.0001$). The three fungi caused significant mortality to the ants (post hoc comparison: *M. brunneum*–control: Wald statistic $z = 3.4$, $p = 0.0008$; *P. lilacinus*–control: $z = 2.3$, $p = 0.02$; *B. bassiana*–control: $z = 2.3$, $p = 0.02$). The mortality caused by *M. brunneum* was significantly higher than the one caused by *P. lilacinus* (Fig. 2, post hoc comparison: Wald statistic $z = 3.8$, $p = 0.0004$), but was just not significantly higher than the one caused by *B. bassiana* ($z = 1.9$, $p = 0.06$). *Paecilomyces lilacinus* and *B. bassiana* did not differ significantly in their virulence ($z = 0.2$, $p = 0.8$).

The survival of ants exposed to three fungal strains of *B. bassiana* and to a control solution differed significantly (Fig. 3, deviance = 29.9, $df = 3$, $p < 0.0001$). Strain S1 had no significant impact on ant survival, as compared to control (Fig. 3, post hoc comparison: Wald statistic $z = 0.4$, $p = 0.64$). In contrast, strains S2 and S3 had a similar and significant negative impact on ant survival (Fig. 3, post hoc comparisons: S2–control: $z = 3.3$, $p = 0.0008$; S3–control: $z = 2.6$, $p = 0.008$; S2–S3: $z = 1.0$, $p = 0.3$). The colony of origin had a significant effect on the survival of ants (deviance = 66.3, $df = 9$, $p < 0.0001$). However, the impact of the fungal strains on ant survival did not depend on the colony of origin of ants, as there was no significant interaction between the two factors (deviance = 36.9, $df = 27$, $p = 0.09$).

Discussion

There are a rapidly growing number of laboratory studies investigating how ants defend themselves against generalist fungal entomopathogens such as species from the *Metarhizium* complex (e.g. Ugelvig et al., 2010; Reber et al., 2011). However, little is known about the prevalence and virulence of fungal pathogens in natural populations of ants. Such data are essential to evaluate the evolutionary relevance of behavioural and immunological studies on host–fungi relationships.

Our search for fungi in soil samples using *Tenebrio* baiting revealed the presence of at least eight species of fungal entomopathogens in the habitat of *F. selysi*. We detected some well-known generalist entomopathogens, in particular *M. brunneum* (a lineage of the *M. anisopliae* complex recently elevated to the species level, Bischoff et al., 2009) and *B. bassiana*. *Aspergillus flavus*, *Mucor hiemalis, P. lilacinus* and *B. bassiana* appeared to be widely

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distributed, each occurring in more than 12% of the samples. Overall, this semi-quantitative survey suggests that *F. selysi* is frequently confronted with spores from a diversity of entomathogenic fungal species while foraging or nesting in its natural habitat.

Our systematic search for signs of infection by *M. brunneum*, *B. bassiana* and *P. lilacinus* in ants from field colonies revealed large differences in the prevalence of the three fungal species at the time of sampling. *Paecilomyces lilacinus* had the highest prevalence, having been detected in 36 of the 81 colonies surveyed, both in dump piles and ants collected alive. The high prevalence of this species in ant colonies is in line with its abundance in soil samples. *Paecilomyces lilacinus* is a common saprotrophic fungus that is known to be pathogenic for root-knot nematodes and various insect species (Tiganomilani et al., 1995; Fiedler and Sosnowska, 2007). *Paecilomyces lilacinus* has recently been isolated from leaf-cutting ant queens, with evidence that it grew from within the ant corpse and caused prolific sporulation (Rodrigues et al., 2010). However, it may also cause direct mortality through toxins (Yang et al., 2007). The frequent occurrence of *P. lilacinus* within and around colonies of *F. selysi*, its presence in four abandoned nests and the significant mortality it caused to workers suggest that *P. lilacinus* may be an important pathogen of ants.

In our snapshot estimate of prevalence, *B. bassiana* was detected in 14 out of 81 colonies. *Beauveria bassiana* is an obligate-killer that infects many insect species (Meyling and Eilenberg, 2007). It was frequent in the soil of our study population. Interestingly, *B. bassiana* was overrepresented in colonies that were also infected by *P. lilacinus*. The frequent co-occurrence of the two pathogens may result from a weakness or general susceptibility of the infected colonies, from environmental conditions favourable to both fungal species before the time of sampling, or from some interactions between the two fungal species. For example one of the two pathogens might take advantage of an ongoing infection by the other pathogen to invade the colony. For example, *Aspergillus flavus*, an opportunistic fungal species of low virulence, sporulated more frequently from corpses of *Acromyrmex echiniator* when co-infected with *Metarhizium* (Hughes and Boomsma, 2004).

Somewhat surprisingly, *B. bassiana* was detected in ants collected alive, but never in dump piles, despite the fact that entire dump piles were collected and that every corpse was assessed for sporulation. The hygienic behaviour of ants may have contributed to inactivate the spores in dump piles, rendering them undetectable. For example, corpses were generally cut into pieces and exposed to sunlight, so that UV light and dryness may impede the development of the fungus (Fernandes et al., 2007; Grijalba et al., 2009). The grooming and spreading of anti-septic substances on the surface of the corpses may also decrease the viability of spores, as has been reported in leaf-cutting ants (Fernandez-Marin et al., 2006; Poulsen et al., 2006). These defences may be more efficient against the obligate-killer *B. bassiana* than against the more opportunistic *P. lilacinus,* which was abundant in dump piles.

We did not find *M. brunneum* within live colonies, although we isolated it from two soil samples, including one from an abandoned nest. The absence of *M. brunneum* in live colonies may simply reflect its rarity in the environment of the ants, particularly if high doses are requested to cause infection (e.g. Hughes et al., 2002; Reber et al., 2008, 2011). In any case, the differences in prevalence among pathogens that we observed at a single point in time should be considered with some caution, as hosts–pathogens are dynamic systems.

There was a high level of genetic diversity among the *B. bassiana* isolates. First, sequence variation in mitochondrial and ribosomal genes delineated three well-differentiated genetic strains. Moreover, the strains were differentiated from one another by multiple, fixed allelic differences at many of the microsatellite loci examined. Second, there was variation in microsatellite genotypes within the two strains for which more than one isolate was sampled. We found 6 haplotypes out of 15 isolates in strain S1, and 2 out of 3 isolates in strain S2. Hence, multiple lineages of *B. bassiana* have colonized the habitat and infect the study population. The fact that the majority of isolates in strain S1 had an identical haplotype is a clear sign of asexual reproduction, and the genetic diversity within strains may be due to an accumulation of mutations in ancient lineages. However, the current data set does not permit us to exclude the possibility that sexual reproduction might occasionally occur (Meyling et al., 2009).

Overall, the three fungal species caused significant mortality to experimentally challenged ants, but varied in their degree of virulence, with *M. brunneum* causing the highest mortality. The three strains of *B. bassiana* also differed in their virulence. The most common strain did not cause any extra mortality to experimentally challenged ants. In contrast, the two rare strains caused significant mortality. The low virulence strain of *B. bassiana* might reflect an adaptation to other host species, as this fungus is an obligate-killer. Alternatively, a decrease in virulence may be associated with vertical transmission and persistence within the long-lived colonies of our study population (Rosset and Chapuisat, 2007), which is in-line with the higher prevalence of this strain in the population (Frank, 1996; Ebert and Weisser, 1997).

This study shows that some fungal pathogens are commonly found in ant colonies. In our field population of *F. selysi*, nearly half of the colonies were infected. However, the sampling effort was large, as we scanned 4050 ants collected alive and entire dump piles from 81 colonies to

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detect fungal infection. Overall, less than 2% of the ants collected alive turned out to be infected. The contrast between the high proportion of colonies harbouring pathogens and the low proportion of infected individuals within colonies is consistent with the idea that ants have effective means to control fungal infections. These defences can be individual or social, and include spatial compartmentalisation, grooming and chemical defences (Chapuisat et al., 2007; Cremer et al., 2007; Wilson-Rich et al., 2009; Reber et al., 2011).

The observed variation in the prevalence and virulence of generalist fungal entomopathogens in ant colonies suggest that the ant hosts and their fungal pathogens interact in specific ways. The prevalence and virulence of pathogens might be negatively correlated in our study population: indeed, the more common pathogens were the opportunistic *P. lilacinus* and the strain of *B. bassiana* of low virulence. In general, a negative correlation between prevalence and virulence is expected, because the more virulent parasites tend to kill their hosts more frequently, which reduces prevalence (Frank, 1996). Such a negative correlation has been documented in birds (Møller et al., 2009). However, this correlation might not occur if the pathogens exploit a wide range of hosts. Overall, more surveys of natural populations of social insects, combined with experiments in the laboratory, will be needed gain a more complete picture of the relationships between prevalence, virulence, specificity and anti-parasitic behaviours.

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