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RESEARCH ARTICLE

Insights into the biodiversity and causes of distribution of potential entomopathogens associated with leaf-cutting ants

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Abstract
To our knowledge, this work is the first large-scaled, systematic survey of potential entomopathogens associated with worker ants of several *Acromyrmex* species. The study was performed at nine sites located in five Phytogeographical Provinces across Argentina. We recorded 28 species of fungi with entomopathogenic behaviour, which infected 24.3% of the 4737 collected ants from 94 colonies. *Fusarium oxysporum* and *F. solani* were the most widely distributed, followed by *Purpureocillium lilacinum* and *Beauveria bassiana*. The occurrence of species across nests within the same site varied from null to 98%. We did not detect any systematic association between fungi and site, Phytogeographical Province or ant species. Instead the microhabitats that surround each nest appear to play an important role in defining entomopathogen communities. We found that climatic variables like maximum temperature, dew point, and relative humidity helped to account for the distribution of these fungi at the site scale. Besides, colonies from undisturbed sites showed higher abundance of infections with entomopathogens than those from disturbed ones. These results greatly improve the knowledge of the ecology of the filamentous fungi associated with leaf-cutting ants. In addition, we proposed that the combination of the entomopathogen virulence and the resistance of ant colonies may be an important but overlooked effect influencing the diversity of entomopathogens.

Keywords *Acromyrmex* · Fungal abundance · Richness · Climatic variables

Introduction

Leaf-cutting ants (Hymenoptera: Formicidae) are well known for the damage they cause to vegetation. They cut leaves, flowers and fruits to cultivate a symbiotic fungus (Agaricales: *Leucoagaricus*) from which they feed (Hölldobler and Wilson 1990). The "higher attines", including *Atta* and *Acromyrmex* species, feed the queen and brood of the colony with gongylidia, a nutrient-rich hyphal swelling, produced by the cultivar (Fisher et al. 1994).

In addition to the ants and the cultivar, other partners are proposed to be part of this symbiosis (Aylward et al. 2012a), playing different roles: *Escovopsis* species, which are specific parasites of the fungal cultivar (Currie 2001); actinobacteria, from genus *Pseudonocardia* specially, which have been described to defend the cultivar from *Escovopsis* (Currie et al. 2003); and a black yeast that parasitizes the filamentous bacteria by acquiring nutrients from it (Little and Currie 2007).

The cultivar, or "garden", grown by higher leaf cutter ants represents an ecosystem in itself, including numerous other microfungi, yeasts and bacteria (Aylward et al. 2012b; Carreiro et al. 1997; Haeder et al. 2009; Pinto-Tomas et al. 2009; Rodrigues et al. 2005, 2008; Scott et al. 2010). Among filamentous fungi, species of the following genera seemed to be the most frequently found in the gardens: *Aspergillus* sp., *Penicillium* sp., *Mucor* sp., *Rhizopus* sp., *Fusarium* sp., *Cunninghamella* sp. and *Trichoderma* sp., as well as *Escovopsis* sp. (Marfetán and Folgarait, in press; Pagnocca et al. 2012). Except for the last one, which was not found anywhere else besides leaf cutter ant nests, the other fungi belong to species commonly found in soil and plant substrates (Watanabe 2002). The mentioned studies have found interesting roles for some of the associated microorganisms, such as fixing nitrogen or decomposing recalcitrant plant tissues. However, these roles are limited to bacterial activity. In contrast, the function(s) of other fungi within and beyond the garden are not as well understood. Most probably, they play

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an opportunistic role either as antagonists of the cultivar or of the ants. Therefore, studying the diversity of fungi associated with the ants both inside and outside the nests will enhance our understanding of broader interactions between leaf-cutter ants and fungi.

As social insects, leaf-cutting ants could be expected to be more susceptible to entomopathogenic fungi because of their group-living; however, few cases are reported in nature (Schmid-Hempel 1998). The absence of disease in natural populations is probably due to "social immunity" (Loreto and Hughes 2016), i.e. a complex collection of behaviours that promote disease control. In leaf cutter ants, the following have been described: cleaning behaviours, both of the ants themselves (Hughes et al. 2002) and their food (Currie and Stuart 2001); removal of infected and dead individuals from the nest (Schmid-Hempel 1998); production of antibiotics and antifungal substances by mutualistic filamentous bacteria (Actinomycetes) (Currie 2001); and fungicidal secretions from the ants' metapleural glands (Fernández-Marín et al. 2006). All these tools could help these ants in the avoidance of entomopathogens as well as pathogens that attack their cultivar. Nevertheless, entomopathogens have been found in abandoned or dead nests (Schmid-Hempel 1998).

Araújo and Hughes (2016) have expressed concern at the lack of publications related to natural diversity of entomopathogens associated with social insects in general. For leaf-cutting ants, there are only few studies which report this kind of data. Ribeiro et al. (2012) found ten entomopathogenic fungi species on *Atta bisphaerica* worker ants. In *Acromyrmex lundii* worker ants, several entomopathogens species were also described naturally infecting them (Goffré and Folgarait 2015), mainly species of *Aspergillus, Fusarium* and *Beauveria* genera. However, the common goal of these studies was to find the best entomopathogen for biological control purposes. In contrast, Hughes et al. (2004) collected 100 worker ants from 5 colonies of *Atta cephalotes, Atta colombica, Acromyrmex octospinosus* and *Acromyrmex echinatior* with the aim of establishing which entomopathogenic fungi the ants were coming into contact with and how often infections appeared to occur. They found that no ants were infected by entomopathogenic fungi; except for three unhealthy workers collected from the dump piles ($N = 20$ for colony) which exhibited infection with *Metarhizium anisopliae* var. *anisopliae*, despite high abundance of this fungus in the area.

In the same way, the present study was designed to broadly survey the occurrence and diversity of potential entomopathogenic fungi collected from foraging ants of different species of *Acromyrmex*, found in several geographical Provinces of Argentina with the goal of improving knowledge of the ecology of these filamentous fungi associated with leaf-cutting ants. We proposed the following hypotheses to explain their diversity. If horizontal transmission is an important factor for entomopathogen spread, we expect to find different patterns of entomopathogen occurrences among sampling sites. We also considered that the occurrence of entomopathogens depends on the ant species because of their particular sanitary behaviours that could be species specific. Finally, entomopathogen presence could also differ among Phytogeographical Provinces because different characteristics and climatic conditions could favour some entomopathogenic species and not others.

Methods

Ant species, fungal isolation and identification

Six leaf-cutting ant species included in the genus *Acromyrmex* were collected: *A. aspersus, A. crassispinus, A. heyeri, A. lobicornis, A. lundii* and *A. striatus*. Nests were located at nine different sites from Argentina, distributed across five Phytogeographical Provinces (Cabrera 1976) (Fig. 1). Below, we provide a brief description of each Phytogeographical Province, all of them included in the Neotropical Region (Cabrera and Willink 1973). Each one represents the geographic areas where typical vegetation grows in relation to a given clime.

The Yungas Province (Amazonian Domain) extends along the eastern slopes of the mountains of north-western Argentina, from approximately 500 to 2500 m in altitude. The climate is warm and humid, mainly with summer rains. The annual precipitation varies from 900 to 2500 mm, and the average temperature between 14 and 26 °C. The predominant type of vegetation is the cloud forest, with trees about 30 m high, abundant lianas and epiphytes, and a very dense lower stratum formed by shrubs and grasses. The Pampas Province (Chacoan Domain) occupies the horizontal plains of eastern Argentina, with some low mountain ranges (up to 1200 m). There are rivers with slow and undulating courses, and numerous lagoons with fresh or brackish water. The climate is warm temperate, with year-round rainfall that decreases from north to south and east to west, from 1100 to about 600 mm annually. The average annual temperature is between 13 and 17 °C. The dominant vegetation is the grass steppe. The Espinal Province (Chacoan Domain) is characterized by plains, lowlands and dunes, under a climate that is warm and humid in the north part, temperate and dry in the west part. The precipitation varies from 340 to 1170 mm and the average annual temperature from 15 to 20 °C. The dominant vegetation type is the xerophilous forest. This province is characterized by the dominance of tree species of the genus *Prosopis*. The Monte Province (Chacoan Domain) features sandy plains, pockets, plateaus and low

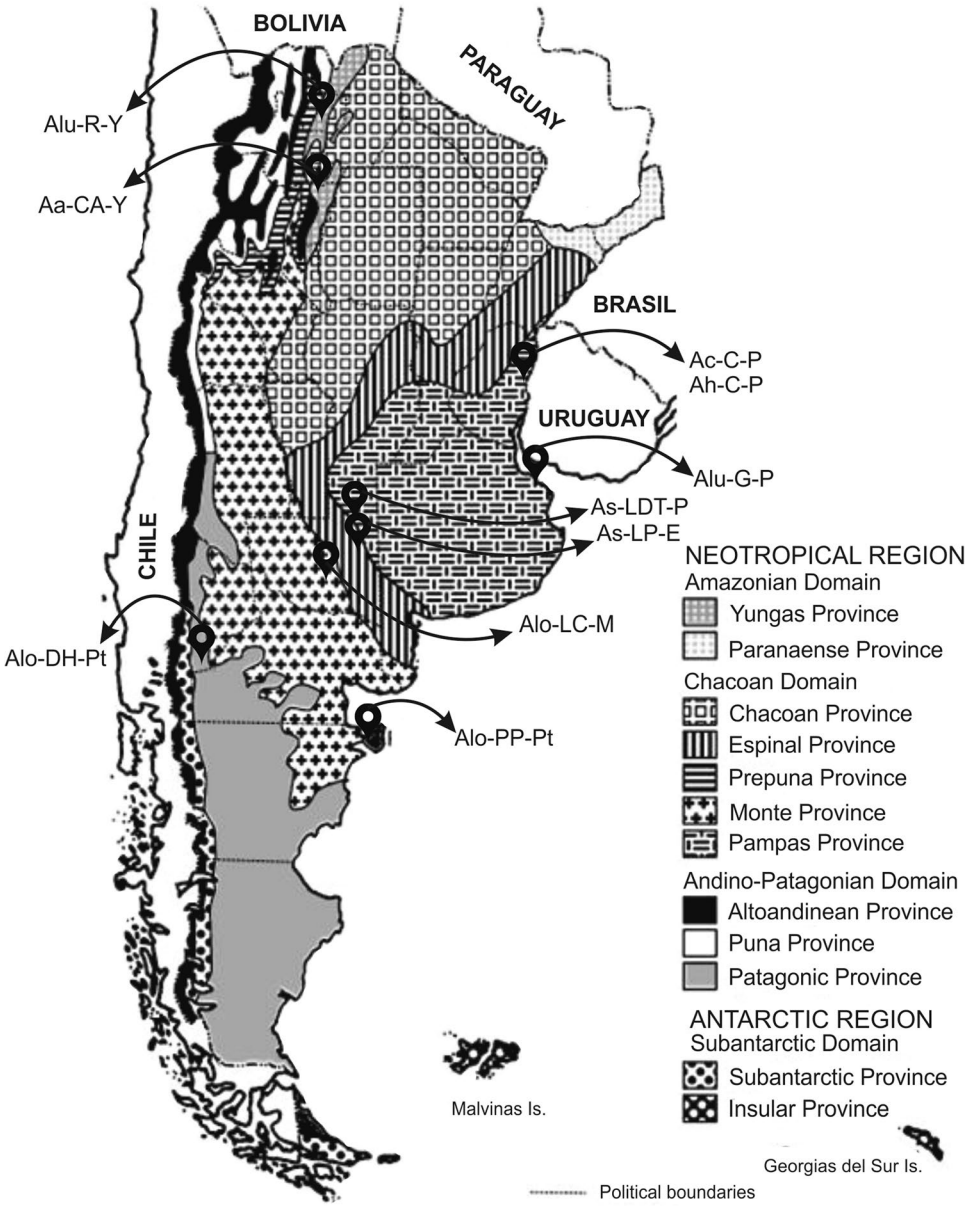


Fig. 1 Phytogeographical Province of Argentina (modified from Cabrera 1976). Each marked point represents each sampling site. Codes indicate, in order: ant species-sampling site-Phytogeographical Province (for details see Table 1)

mountain slopes, with a dry and warm climate in its northern part, dry and cool in the south. The precipitation varies between 80 and 250 mm annually, rarely more; and the average annual temperature between 13 and 17.5 °C. The predominant type of vegetation is the xerophilic, psamophile or halophilous shrub or steppe shrub. There are also marginal forests of carob or willow. The Patagonic Province (Andino-Patagonian Domain) is characterized by plateaus and low mountains with sandy-stony skeletal soils, under a dry and cold wind-swept climate, heavy snowfall during the winter and frost almost all year round. The average temperature varies from 13.4 °C in the north, to 5 °C in the south; the precipitation oscillates between 100 and 270 mm annually, increasing up to about 500 mm in the western edge of the Province. The dominant vegetation is the shrub steppe, with broad-leaved, narrow or spiny leaves, or with predominantly cushion species.

Each sampling site was identified with a code, which refers to the ant species, the name of the site, and the Phytogeographical Province (Table 1). We collected the most abundant *Acromyrmex* species present at each site. At Concordia, we included both *A. crassipinus* and *A. heyeri*, and thus used a total of ten site-species combinations. We always tried to find at least six nests per site, except at Puerto Pirámides, where we had logistical problems that prevented us from staying longer.

For each nest, 45–55 ants were collected from cutting and foraging trails with forceps or manually, wearing latex gloves. Implements were disinfected with ethanol 70% every time, samples from a new colony were collected, and ants

Table 1 Codes for each sampling site, starting with the ant species, and followed by the name of the site, the Phytogeographical Province and coordinates

Code	Ant species	Site	Phyto-geographical Province	Coordinates
As-LDT-P	*A. striatus*	Laguna Don Tomás	Pampas	−36.61036 −64.31284
As-LP-E	*A. striatus*	Provincial Reserve Luro Park	Espinal	−37.19431 −64.04987
Aa-CA-Y	*A. aspersus*	Campo de Alisos National Park	Yungas	−27.2917 −65.86303
Alu-R-Y	*A. lundii*	Rey National Park	Yungas	−24.6701 −64.60098
Alu-G-P	*A. lundii*	M. B. Gonnet	Pampas	−34.8772 −58.01562
Ac-C-P	*A. crassipinus*	Concordia	Pampas	−31.66302 −58.02394
Ah-C-P	*A. heyeri*	Concordia	Pampas	−31.66302 −58.02394
Alo-LC-M	*A. lobicornis*	Lihué Calel National Park	Monte	−37.9626 −65.59112
Alo-PP-Pt	*A. lobicornis*	Puerto Pirámides	Patagonic	−42.57156 −64.27197
Alo-DH-Pt	*A. lobicornis*	Dina Huapi (Nahuel Huapi National Park)	Patagonic	−41.07365 −71.15776

were placed together in a 50 ml tube, maintained in darkness. Once in the laboratory (after 48–72 h) ants were put individually in humid chambers and, after their death, cadavers were inspected every 2 days looking for any fungal growth. None of the ants appeared infected before being placed in the humid chambers. No disinfection was carried out in order to collect as much information as possible in each sampling. Entomopathogenic growth was considered only if it appeared from inside the ants at intersegmental membranes and/or at the joints of the legs and antennae, which is typical of most entomopathogenic fungi belonging to Ascomycota phylum and some fungi belonging to "Zigomycetes" (Araújo and Hughes 2016). Other fungal growth was considered external and, therefore, non-entomopathogenic.

Only fungi considered entomopathogenic were identified at the species level based on morphological characters observed under the microscope (Nikon model Eclipse E200) and using taxonomic keys (Barnett and Hunter 1998; Carrillo 2003; Watanabe 2002). To identify isolates, we employed overall colony morphology in Potato Dextrose Agar (PDA) as well as morphology of diagnostic characters. For some groups of fungi, we used specific keys (Leslie and Summerell 2006; Pitt and Hocking 2009; Zheng and Gui-qing 2001).

For five of the site-species combination (Ac-C-P, Ah-C-P, Alu-G-P, Alo-DH-Pt and Alo-PP-Pt), survivorship was measured every other day until the death of all ants. To conduct a preliminary evaluation whether the entomopathogens found affect ant survival, we calculated the mean survival time for ants that died due to each entomopathogen species found and compared them to the mean survival time of ants from the same colony that died from no apparent reason ("non-infected" ants).

Analysis and description of the entomopathogen community

First, we estimated theoretical richness, using estimators of EstimateS 6.0 program (Colwell 2005) and the Clench function adjusted with minimum mean square to obtain the theoretical maximum richness for entomopathogens at the site scale (Moreno 2001). To maintain the nests as independent sampling units, isolates from the same entomopathogenic species obtained from the same nest were counted as 1 no matter in how many ants it was present. We evaluated the efficiency of our sampling effort comparing the richness found per site against the maximum theoretical richness. We considered a sample satisfactory when at least 70% of the theoretical richness was represented.

In most nests, we found more than one species, allowing us to define a community of entomopathogens for each nest. Communities´ composition and abundance were compared using a two-way cluster analysis, which allowed the grouping of nests based on the frequency of fungi found (left clusters). The analysis performed at the nest scale allowed us to see if a particular entomopathogen community was associated with the ant species, the site or the Phytogeographical Province. Additionally, this analysis also grouped

fungal species according to the frequency of occurrence in each nest sampled (top clusters). For this analysis, we used a frequency matrix of the fungal species isolates as columns and nests as rows. The following parameters were used: Sorensen's distance coefficient as the distance method; and flexible beta as the linkage method with $\beta = -0.9$ for the nest scale, to avoid highly chained dendrograms (McCune and Mefford 2011). Boxes within the matrix were coloured in different shades of grey according to the frequency of infected ants per nest by each fungal species.

The multivariate analysis was performed using PcOrd 6.0 (McCune and Mefford 2011).

Fungal responses to climatic variables

A correspondence analysis (CA) was performed to evaluate how the entomopathogen species were ordered in space according to orthogonal axes based on their frequency for each site. We built a matrix with fungal frequency as columns and sites as rows. Since the amount of variance explained was important and the first axis was significant, we performed additional analyses. The first of these consisted of a principal components analysis (PCA) to identify the best and least correlated climatic variables to use as a secondary matrix (climatic data as columns and sites as rows) in a canonical correspondence analysis (CCA). We started with 22 climatic variables and chose the best 8 ones (greater Euclidean distance in the first two eigenvectors, lower correlation among themselves, and unimodal distribution, which would be required for subsequent analysis).

We obtained annual, monthly and daily climatic data from meteorological stations located near the sampling sites from the Meteorological National Services of Argentina, Freemeteo (2016) and National Institute of Agricultural Technology (INTA 2016). When data were not available for each sampling site, we used that from the closest monitoring station (not farther than 100 km). Data included temperature, relative humidity, dew point (temperature at which airborne water vapour will condense to form liquid), atmospheric pressure, maximum wind, and precipitation. We took into account climatic data from exactly the same day when samples were collected at each site, as well as the averages for the month and year, defined as 30 and 365 days before the sampling day, respectively.

The second additional analysis was the CCA to test whether climatic variables might help explain the variation observed in the distribution of entomopathogens (Jongman et al. 1995; Ter Braak 1986), using fungal frequency per site (primary matrix) and selected climatic variables (secondary matrix). Axis scores were entered and standardized to unit variance and axes scaled to represent the interdependence of fungal species and sampling sites. To interpret the ordination of axes, we used the canonical coefficients (hereafter: SCC) and the intraset correlations (intraset-C). The latter refers to the correlation between the environmental variables and the ordination axes and is quite stable in comparison to the canonical coefficients (Ter Braak 1986). We also reported correlations between sample scores for axis derived from the species data and the sample scores that were linear combinations of the environmental variables (Pearson correlation).

All multivariate analyses were performed using PcOrd 6.0 (McCune and Mefford 2011).

Results

Fungi isolated and their abundance

From a total of 4737 ants, 24.3% were infected with entomopathogenic fungi. The 1151 entomopathogens obtained included 28 species, belonging to 7 genera of Ascomycota: *Fusarium, Beauveria, Purpureocillium* (hereafter: *Pu.*), *Aspergillus, Penicillium* (hereafter: *P.*), *Metarhizium* and *Paecilomyces* (hereafter: *Pa.*); and 1 belonging to Zygomycota: *Cunninghamella*. Only *F. solani* and *F. oxysporum* were found in all sites, and *Pu. lilacinum* and *B. bassiana* in many of them (from here on, these four species are referred to as common entomopathogens). From the remaining species, 16 were rare and 8 were singletons, i.e. they were found in only one ant of one nest in only one site: *P. fellutanum, P. restrictum, P. raistrickii, P. phoeniceum, P. citrinum, M. anisopliae, A. ustus* and *F. semitectum*.

There was great variability in the abundance of entomopathogen species among sites (Table 2). We found that *A. aspersus* ants from Tucumán in Yungas Province (Aa-CA-Y) were the most infected with entomopathogens (63% of a total of 653 ants across 13 nests), whereas *A. crassispinus* ants from Concordia in Pampas Province (Ac-C-P) and *A. lobicornis* from Puerto Pirámides in Patagonic Province (Alo-PP-Pt) had the lowest infection abundances (7% of 543 ants across 10 nests and 8% of 189 ants across 4 nests, respectively). All sites located in Pampas Province had low abundances (from 7 to 14%). The remaining sites had values ranging from 24 to 41% of infection.

There was no relationship between abundance and richness of entomopathogens; e.g. the site with the highest abundance showed the lowest richness (Aa-CA-Y) whereas the sites with least abundance had intermediate richness (Table 3). Furthermore, nests from the same site had enormous differences in the abundance of entomopathogens species. For example, in the site where the highest abundance was found (Aa-CA-Y), the abundance per nest ranged from 2 to 98%. Similarly, in one nest of *A. crassispinus* from Concordia (Ac-C-P), 7 out of 51 ants were infected only with *Pu. lilacinum*. In another nest, 5 out of 54 ants were infected with *F. oxysporum* and *F. semitectum* and no ants infected

Table 2 Nests sampled and total number of ants for each site are presented, followed by the totals of ants with fungi (entomopathogenic or non-entomopathogenic) or ants without fungi. The abundance of entomopatogens (as median with quartiles) is also shown

	As-LDT-P	As-LP-E	Alo-LC-M	Aa-CA-Y	Alu-R-Y	Ac-C-P	Ah-C-P	Alo-PP-Pt	Alu-G-P	Alo-DH-Pt
Nests sampled (*N*)	10	9	9	13	13	10	10	4	7	9
Total of ants	491	431	447	653	632	541	539	188	361	447
Entomopathogenic fungi	80	116	166	339	183	39	44	18	51	124
Non-entomopathogenic fungi	111	99	175	142	67	243	335	53	174	275
Without fungi	300	216	106	172	382	259	160	117	136	48
Abundance of entomopathogens (median and quartiles)	8 (4–18)%	30 (23–34)%	41 (30–42)%	63 (25–74)%	24 (14–54)%	7 (5–9)%	10 (1–11)%	8 (6–12)%	14 (8–22)%	25 (19–30)%

Table 3 Richness found for each site, maximum theoretical richness obtained, and percentage of observed richness relative to the maximum (for codes refer to Table 1)

	Richness/site	Maximum theoretical	Percentage represented (%)
Alo-DH-Pt	11	16	69
Alo-LC-M	10	12	83
As-LP-E	9	10	90
Alu-R-Y	9	11	82
Ac-C-P	7	10	70
Ah-C-P	6	10	60
As-LDT-P	6	8	75
Alu-G-P	5	13	38
Alo-PP-Pt	4	8	50
Aa-CA-Y	3	3	100

with *Pu. lilacinum*. Meanwhile, ants from another nest had no entomopathogenic fungi. The remaining sites exhibited similar patterns.

In addition, we performed a preliminary evaluation of mean survival time for ants that died due to each entomopathogen species compared to the mean survival time of ants from the same colony that died from no apparent reason ("non-infected" ants), for each colony. This could only be done for 5 from the 10 site-species combinations (overall 40 nests). Common entomopathogens, such as *F. oxysporum, F. solani* and *P. lilacinum*, were associated with lower mean survival times than the "non-infected" ones, in 52.4, 62.5 and 64.3%, respectively, of all the colonies where they were present. In the case of rare or singletons species, *M. anisopliae*., *F. semitectum, Pa. niveus, A. fumigatus, P. restrictum, P. citrinum, P. simplicissimum* and *B. bassiana* produced a negative effect on survival time compared to those ants which died for other reasons. In contrast, for the remaining species, infected ants survived similarly or longer than their non-infected nestmates (those infected with *A. ustus, A. oryzae, Cunnighamella* sp., *F. chlamydosporum, Fusarium* sp., *P. verrucosum, P. raistrickii, P. waksmanii* and *P. phoeniceum*).

Analysis of entomopathogen communities

Three out of the ten site-species combinations did not meet the condition of representing at least 70% of the theoretical richness, although only *A. lundii* from Gonnet (Alu-G-P) was grossly underestimated (only a 38% of the theoretical richness was sampled) (Table 3).

We performed the multivariate analysis only with sites that met our criteria of being well sampled (70% or more of theoretical richness), and then compared with the results obtained with all the sites together. We did

not find differences between the formed clusters; therefore, we presented the results with all the sites. For the latter, we used the 86 nests which had infections with entomopathogens; nests without entomopathogens were not considered (Fig. 2).

We expected to observe an association between entomopathogenic species and ant species, sampling site and/or Phytogeographical Province. No single factor could explain the species clusters observed at the nest level (Fig. 2). Ants of the same species did not group together; for example, *A. lundii* was recorded at several sites and Phytogeographical Provinces and was spread throughout the groups (Fig. 2). Although we observed that nests from Dina Huapi (Alo-DH-Pt) were clustered together, the same did not happen with all the other sites. In some cases, Phytogeographical Provinces were grouped; e.g. 10 out of 30 nests from Pampas Province (P in Fig. 2), 13 out of 26 nests from Yungas Province (Y) and 8 out of 13 nests from Patagonic Province (Pt). However, there was a partial agreement and only in three of the five Phytogeographical Provinces. Based on these results, we concluded that communities of entomopathogens should be defined at the scale of individual nests.

With respect to entomopathogen frequencies within nests, only *F. oxysporum* and *F. solani* showed frequencies per nest higher than 0.5 in few cases, mainly in Yungas Province (Fig. 2). *Pu. lilacinum* first and *B. bassiana* second were the following species with highest frequency per nest and found in several nests. *F. poae, Fusarium* sp., *C. echin*. var. *echin., C. septata, C. echin*. var. *vert*. and *Pa. niveus* exhibited frequencies in the range of 0.49–0.1, at least once, whereas the other rare species had always frequencies lower than 0.09, which it means fewer than five ants per nest infected with these fungi species.

Furthermore, we observed that each nest showed different richness of entomopathogens and none of the species appeared in all the nests of the same site (Fig. 2). One or 2 entomopathogen species appeared in 43 nests, 3 or 4 species in 31 nests, 5 or 6 species in 12 nests, and only one nest (from Alo-LC-M) presented 8 species. The 8 nests that did not have entomopathogenic fungi (3 from As-LDT-P, 3 from Ah-C-P and 2 from Ac-C-P) were all from the Pampas Province.

Only two fungal species were widely distributed across nests sampled (*F. oxysporum* and *F. solani* appeared in 64% and 70% of the nests, respectively). Few of them exhibited an intermediate distribution (*Pu. lilacinum* and *B. bassiana* present in 36% and 25%, respectively), whereas the majority were present only at one or two nests (Fig. 2).

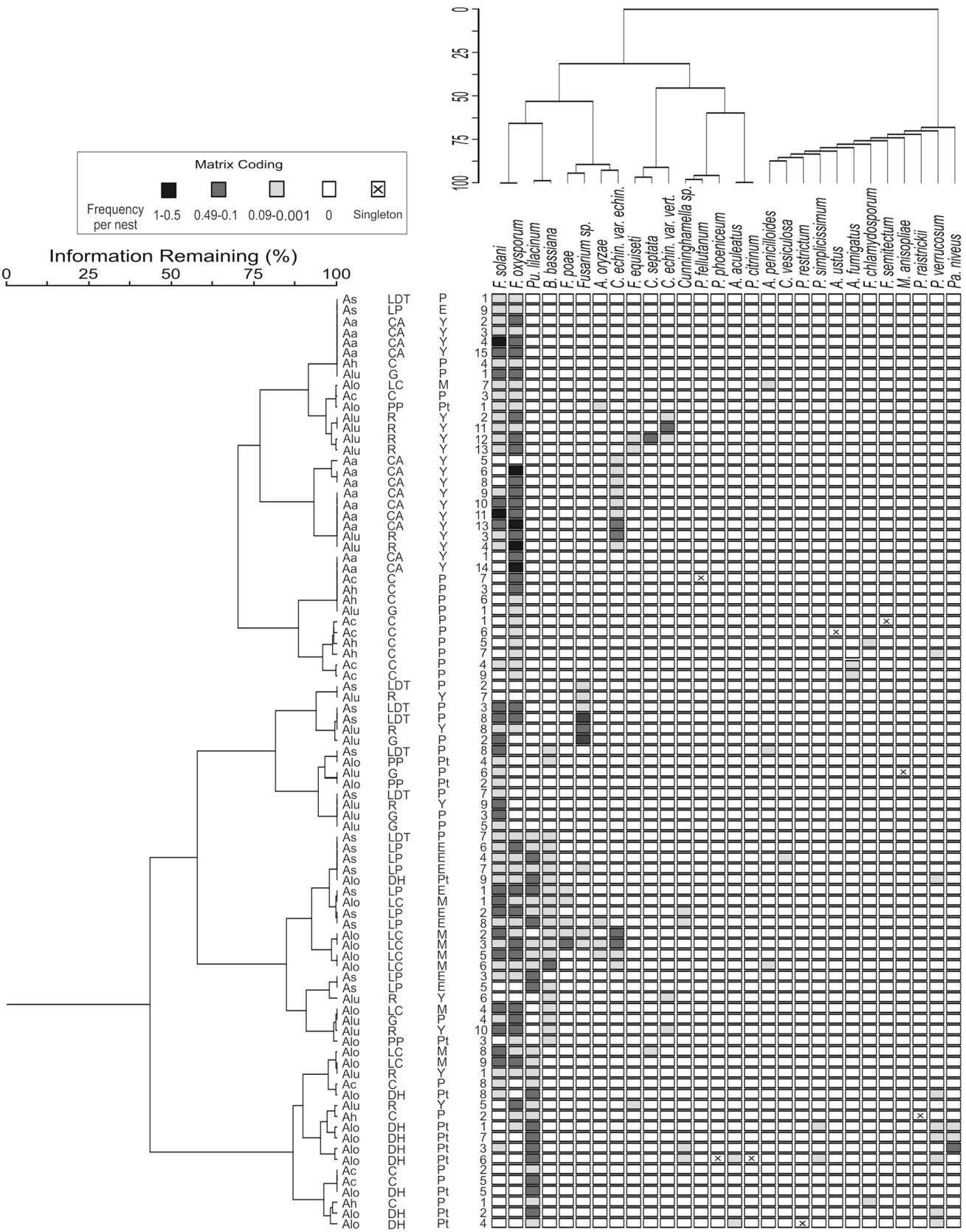
The relationship between fungal species and climatic variables

We analysed the abundance of entomopathogens species in relation to the environmental conditions at the site scale. Although we attempted to use the nest scale for running this analysis, the randomization test failed to shuffle data because the matrix was very sparse (many zero values) due to the presence of rare species and singletons (McCune and Mefford 2011). Therefore, we assembled the data at the next higher level which was the site.

The correspondence analysis of fungal species at site scale allowed us to explain 58.7% of the variance with the first two axes, and we defined three groups. The first and the largest group contained most of the sites (Alu-R-Y, Aa-CA-Y, Alu-G-P, As-LDT-P, Alo-LC-M, Alo-PP-Pt) and mostly common entomopathogens (*F. oxysporum, F. solani, B. bassiana*) plus some rare species. The second group contained only Alo-DH-P, where *Pu. lilacinum* (a common species) predominated along with rare and singleton species. The third group was formed by rare and singleton species, and was ordered mostly in relation to axis 2. We verified that singletons did not modify the pattern found, running this analysis only with common and rare species (results not shown).

Following that, we conducted a PCA with climatic variables. Environmental differences across the sites were explained using a matrix with the following selected variables: annual average maximum temperature (AAMAXT), monthly average maximum temperature (MAMAXT), annual average relative humidity (AARH), monthly average relative humidity (MARH), dew point of the sampling day (DPSD), atmospheric pressure of the sampling day (APSD), average wind of the sampling day (AWSA), and monthly average wind (MAW). These variables were uncorrelated to each other (lower than $r = 0.8$) and showed unimodal distributions. A total of 70.6% of the variance was explained by the first two axes of the PCA analysis. The first axis was represented mainly by greater temperature (MAMAXT), higher dew point (DPSD) and more relative humidity (AARH) towards the positive side of the axis, and windy sites towards the negative side (AWSD and MAW). The second axis showed higher maximum temperature (AAMAXT) ordered towards the positive side of the axis, and higher atmospheric pressure (APSD) to the negative side.

Finally, we performed a CCA to test whether climatic variables might help explain the variation observed in the ordination of entomopathogens. The analysis showed that the cumulative variance for the first two axes was 57.2%, in which the first and second axis explained 42.1 and 15.1%, respectively (Fig. 3). Pearson correlation was $r = 0.998$ for each of both axes.



◂**Fig. 2** Two-way cluster analysis of the fungal composition at the nest level. Row codes (see Table 1) indicate, in order: ant species-sampling site-Phytogeographical Province-nest number. Abbreviations correspond to *C. echinulata var. echinulata* (*C. echin. v. echin.*) and *C. echinulata var. verticillata* (*C. echin. v. vert.*). Matrix coding: black when the frequency per nest of the fungal species is equal or greater than 0.5; dark grey when it is from 0.49 to 0.1, light grey when it is lower than 0.09 but not zero; white represents absence and X shows singletons (those that appeared in only one ant of one nest in only one site)

The ordination obtained through CA and CCA did not differ much, so we inferred that the measured environmental variables accounted for the majority of the variation in the species data (Ter Braak 1986). Important environmental variables were AAMAXT (SCC = 1.271, intraset-C = 0.831) and DPSD (SCC = 1.271, intraset-C = 0.621) in relation to the axis 1, and AARH (SCC = − 1.832, intraset-C = − 0.785) and MAMAXT (SCC = 1.812, intraset-C = − 0.650) related to the axis 2.

In the CCA (Fig. 3), relevant groups of species were highlighted within a circle: one group of species associated with low annual maximum temperature at the left upper quadrant, and three groups of species at the right quadrant with higher annual maximum temperature but associated with lower, intermediate and higher humidity at the upper, middle and lower right quadrant, respectively. This analysis revealed that common entomopathogen species were found at all climate conditions and sites. The right side of the first axis was represented by sites with higher annual maximum temperatures (higher than 20 °C) and higher dew point (more than 13 °C). *Pu. lilacinum, Cunninghamella* sp. and some rare and singleton species were associated with the left side, i.e. low annual maximum temperatures (lower than 20 °C), as it was found in Dina Huapi (Alo-DH-Pt).There also seemed to be a gradient of relative humidity (range 50–80%) in relation to the second axis. Species such as *F. chlamydosporum, A. fumigatus* and other singleton species were related to higher humid sites (over than 75%) and higher monthly maximum temperature (higher than 30 °C), characteristics which are typically associated with the Concordia site, in which these species were found (Ac-C-P and Ah-C-P). *F. equiseti, C. echinulata* var. *echinulata* and *C. septata* were more related to intermediate humid areas (between 60 and 75%). *B. bassiana, A. oryzae, A. penicilloides, F. poae* and *P. verrucosum* seemed to be species which shared tolerance to lower humidity (below 55%). In general, singletons species were distributed mainly on extreme sides of the two axes.

Discussion

This work represents the first record of culturable fungi with entomopathogenic behaviour associated with several *Acromyrmex* species from different sites. The potential entomopathogens we observed include species commonly found in soil and plant substrate (e.g. *Fusarium* and *Penicillium* species; Watanabe 2002), as well as species previously associated with these ants inside the fungus gardens (Pagnocca et al. 2012; Marfetán and Folgarait, in press) or reported as entomopathogens of leafcutter ants (Ribeiro et al. 2012; Goffré and Folgarait 2015).

The results obtained in this study highlight the importance of different aspects that seemed to explain the diversity of potential entomopathogens associated with *Acromyrmex* leaf cutter ants. First, the relevance of the microhabitat in the presence of a particular species in a site; second, the importance of maximum temperature and relative humidity in the distribution of entomopathogens and, finally, the proposed effect of two overlooked factors such as the immunological resistance of the ant colonies and the degree of virulence of different fungal strains to determine the success in killing the host.

We were only able to compare our results with the information for *Atta* workers from Brazil and Panama. Ribeiro et al. (2012) found three species of *Aspergillus* (*A. niger, A. ochraceus* and *A. sclerotiorum*), two of *Penicillium* (unidentified), two of *Mucor* (*M. hiemalis* and *M. racemosus*), one of *Cladosporium* (unidentified), *Purpureocillium* (*Pu. lilacinum)* and *Beauveria* (*B. bassiana)* on *A. bisphaerica* worker ants, which closely match our data on *Acromyrmex*. Taken together, these findings seem to imply that entomopathogens do not segregate based on either ant genus or species. Moreover, Hughes et al. (2004) were unable to find any entomopathogens in *A. cephalotes, A. colombica, Ac. octospinosus* and *Ac. echinatior* nests from Panama sites. These results indicate that apparently entomopathogens do not seem to have co-evolved with the two genera of higher attine ants.

Hypotheses associated with fungal diversity

We found extensive variability in diversity across all levels studied, and we were not able to find significant associations at the level of site, Phytogeographical Province, or ant species. Instead, we defined the entomopathogenic communities at the nest level. Our data showed two patterns. On one hand, only two species, *F. oxysporum* and *F. solani*, were widely distributed; the great majority of species were rare or singletons, even for those sites where the sampling effort detected 70% of the expected theoretical richness. On the other hand, a marked low abundance of entomopathogens, less than 15%, in all sites located in Pampas Phytogeographical Province including several nests without entomopathogenic fungi, despite exhibiting intermediate richness.

It is interesting to note that sites with low abundances matched disturbed sites: Puerto Pirámides plus all sites

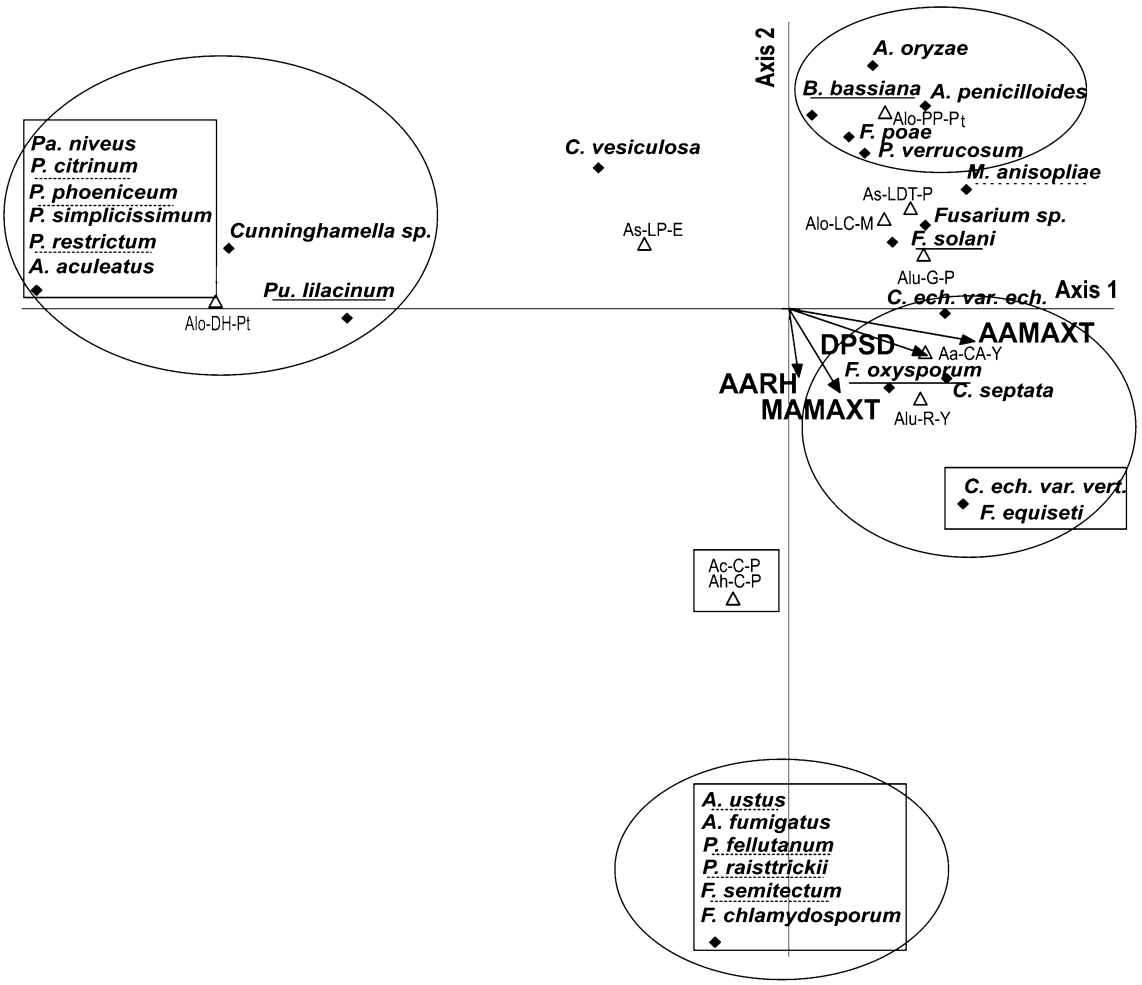


Fig. 3 Joint plot from canonical correspondence analysis. Frequencies of entomopathogen species of leaf-cutting ants, explained by climatic variables. Analysis done by site. Squares show species (or sites) that corresponded to the same point in space, and otherwise cannot be seen. Fungi species are indicated by diamonds whereas triangles show sites. For sites´ codes refer to Table 1. All species named in italics; common entomopathogens (the four first species of the two-way analysis) are underlined with solid lines, singletons with dashed lines, and rare species not underlined. Abbreviations correspond to *C. echinulata var. echinulata* (*C. echin. v. echin.*) and *C. echinulata var. verticillata* (*C. echin. v. vert.*). Climatic variables are average annual maximum temperature (AAMAXT), mean dew point for the sampling day (DPSD), monthly average maximum temperature (MAMAXT), and annual average relative humidity (AARH). Relevant groups were circled

from Pampas Province, which are located on road sides, city squares, and forest plantations; in contrast to those sites in National Parks or Provincial Reserves, e.g. Campo de Alisos National Park, Rey National Park, Lihué Calel National Park, Luro Park and Dina Huapi (the latter inside the Nahuel Huapi National Park). Probably, disturbed habitats are ecologically simpler than undisturbed ones and, therefore, fewer entomopathogens would be found, and/or would have different climatic conditions (e.g. low humidity in the former). This is coherent with the finding of Barker and Barker (1998), who demonstrated that forest plantations and croplands had a low abundance of entomopathogenic fungi infecting insects compared to undisturbed pastures; and Evans (1974), who also showed higher incidence of disease caused by entomopathogens in undisturbed sites in comparison with exploited forests due to the high humidity in the former. One prediction of the disturbance hypothesis is that intact habitats would favour the preservation of entomopathogens, which in turn would keep or reduce the colonies' carrying capacity for those areas. The loss of this balance could be another partial explanation as to why ants become pests in disturbed areas.

Within-site variability

We also detected great variability in the abundance and richness of fungi within each site. For example, the site with the highest abundance (As-CA-Y) showed a range of abundance

per nest from 2 to 98%. In view of this, it is suggested that the microhabitat that surrounds each nest plays an important role as an alternative explanation for the diversity of entomopathogens that could infect soil insects, such as leaf-cutting ants. The observed differences could be associated mainly with the difficulty for the pathogen to "find" and infect a host, especially considering their quick disintegration in the environment (Meyling and Eilenberg 2007). However, it must be taken into account that the presence of an entomopathogen does not imply that it will kill the host, especially because the virulence of each isolate could be different. In addition, other factors could be important in the persistence of these fungi; for instance, competition among strains (Hu and St. Leger 2002) and the dispersal ability of the infective stage of a pathogen (Anderson and May 1981), using abiotic factors such as wind or rain (Inglis et al. 2001), or biotic ones like other animals used as vectors (Dromph 2003; Feng et al. 2004).

Effect of environmental characteristics on entomopathogen distribution and abundance

There are three main known environmental factors that influence the distribution, abundance and stability of entomopathogens: ultraviolet light, temperature and humidity (Vega and Kaya 2012). Given the absence of data, ultraviolet light was not included in this work, but the other two factors were used as possible explanatory variables in our analyses. We observed that the distribution of the most abundant entomopathogens did not seem to be related to any environmental variable, because they were distributed all across the sites, while the other species appeared on account of two main reasons: one associated with annual maximum temperature, and the other related to annual relative humidity. Singleton species, except *M. anisopliae*, appeared in sites with low maximum temperature or with higher humidity.

Our study clearly showed that some of the potential entomopathogens found are not only species from temperate climates (Cabanillas et al. 1989; Pitt and Hocking 2009), but also seemed to be more tolerant to cold temperature, like for example *Pu. lilacinum, Cunninghamella* sp., *A. aculeatus* and some *Penicillium* species. In addition, some species tolerated low relative humidity and, for example, *B. bassiana* needs higher humidity to germinate (Walstad et al. 1970), so its germination and survivorship in other "non-optimal" conditions could be associated with a great physiological capacity.

Possible effects of host resistance and strain-specific virulence

The immunological status or resistance of each colony is another factor that could be related to the abundance of entomopathogens found in leaf-cutting ants. It has been observed that ants possess the ability to overcome infections, even when they are exposed to high levels of conidia in their surroundings; in *A. cephalotes, A. colombica, Ac. octospinosus* and *Ac. echinatior*, it was observed that the ants collected were not infected by any entomopathogenic fungi despite the high abundance of *M. anisopliae* var. *anisopliae* in the proximity of nests (Hughes et al. 2004). It is known that ants' antibiotic excretions are effective in protecting them against this fungus and others (Hughes et al. 2002; Poulsen et al. 2002). In accordance with that, in the present work *M. anisopliae* only appears in one nest ever. One reason for that could be that leaf-cutting ants are well prepared to fight against this species and not that well to defend themselves against other entomopathogens, probably because the evolutionary pressures of obligate entomopathogens (as *M. anisopliae* seemed to be) might be stronger than those of opportunistic or not obligate ones. However, there is no agreement regarding the specialized status of *Metarhizium* with leaf-cutter ants (Loreto and Hughes 2016). In fact, the latter study offered an alternative hypothesis related to a possible resistance acquired by the colony vertically: princesses (future queens) that survive and establish in the field could have the ability to overcome diseases of particular entomopathogens, and that could also explain the different responses of colonies to the exposure of conidia in laboratory tests.

The presence of some fungal species with previously unreported entomopathogenic activity in leaf cutter ants, like those in the genera *Cunninghamella* (*C. vesiculosa, C. echinulata* var. *echinulata, C. echinulata* var. *verticillata, C. septata* and one species unidentified) and *Penicillium* (*P. fellutanum, P. phoeniceum, P. citrinum, P. restrictum, P. simplicissimum, P. raistrickii* and *P. verrucosum*), could also be related to an opportunistic strategy when the host did not offer any resistance against infections. These fungi are part of the mycobiota associated with leaf cutting ants (Marfetán and Folgarait, in press; Pagnocca et al. 2012; Rodrigues et al. 2008), so it could be expected that these species present pathogenic characteristics when the host's immune defences decrease for other reasons. An example was described by Hughes and Boomsma (2004) where the opportunist pathogen *A. flavus* became virulent after being inoculated jointly with *M. anisopliae*, it seemed that the latter inhibits the host's immune defences which would otherwise prevent infections by *Aspergillus*.

At the same time, the degree of virulence of each strain of entomopathogens could also explain their presence in some colonies and not in others. The differential effect on mortality of ants from different colonies that die due to the same entomopathogen species allows us to propose that the degree of virulence seemed to be related to the strain and not to the species (i.e. for *F. oxysporum, F. solani* y *Pu.*

lilacinum). Strain virulence dependency was found in *Escovopsis weberi*, a pathogen that co-evolved with the fungal cultivar of leaf cutter ants (Marfetán et al. 2015). In addition, it is known that some entomopathogenic fungi could be very plastic, for example some isolates of *Fusarium* species are facultative entomopathogens whereas others are obligate; furthermore, few isolates were found to be pathogenic to plants and insects (Teetor-Barsch and Roberts 1983).

Cunninghamella species found in this study were tested in the laboratory against *A. lundii* ants and no negative effects were found on ant survival in more than few colonies (data not shown). Thus, for this genus, it is possible that the isolates chosen had a different effect depending on the ant colony because their degree of virulence is not high enough to kill ants of all colonies, and/or because the colonies were resistant enough to overcome the disease. However, in the case of this genus, in accordance with the results obtained in this study, it is probable that temperature and humidity conditions used in the laboratory test may not have been the correct ones.

Therefore, if one species of the entomopathogens found in this study was considered biological control agent, our results would oblige us to choose which isolates produce a negative effect on ant survival in all or most tested colonies, as a way to overcome the host resistance. It follows that new candidates of control will not appear effortlessly and we need to persist in the bioprospection of these fungi, and then proceed with survival tests against leaf-cutting ants from different colonies, in order to classify each isolate as a suitable or not biological control agent.

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