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Diversity of mycobiota in colonies of different species of leaf-cutting ants and sampling sites across Argentina

Jorge A. Marfetán[1,2,✉] & Patricia J. Folgarait[1,2]

[1]*Laboratorio de Hormigas, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes. Buenos Aires, Argentina.*[2] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Argentina.*

Abstract. Attine ants are a monophyletic group comprising more than 230 species, distributed exclusively in the New World. All higher Attini ants depend on the cultivation of fungus gardens for food, and those gardens are continuously exposed to alien microorganisms. The present work describes, for the first time, the composition, relative frequency and the richness of the fungal species comprising the mycobiota from the garden of the most abundant *Acromyrmex* species from different regions of Argentina. We also compared, using a multivariate approach, the mycobiota composition with the purpose of testing two hypotheses: 1) whether mycobiota is defined by the ant species, or 2) whether mycobiota is determined by the geographical region. We found that each fungal community had a particular composition and abundance: the species that were highly frequent in a community or site, could be rare or altogether absent in another community or site. Species richness, as well as the theoretical maximum richness, also changed with locations. Furthermore, we found that different ant species at the same site had similar mycobiotas, whereas the same ant species in distant locations had only a few fungal species in common. Therefore, we concluded that mycobiota composition changed considerably with geographical site and was not dependent on the ant species. Our results provide an additional understanding of the leaf cutting-ants system, confirming that the garden of leaf cutting ants nests is a complex and dynamic fungal community which is dependent on the site where it was located.

[Keywords: abundance, *Acromyrmex*, Ascomycota, fungal communities, richness, Zygomycetes]

Resumen. Diversidad de micobiota en diferentes especies de hormigas cortadoras de hojas y sitios de Argentina. Las hormigas de la familia Attini son un grupo monofilético que incluye más de 230 especies distribuidas exclusivamente en el Nuevo Mundo. Todas las hormigas Attini superiores se alimentan de un hongo que cultivan dentro de sus nidos, y los cultivos de este hongo se encuentran continuamente expuestos a microorganismos exógenos a la colonia. Este trabajo describe por primera vez la composición, la frecuencia relativa y la riqueza de las especies que conforman las micobiotas encontrada en los jardines de las especies del género *Acromyrmex* más abundantes en diferentes regiones de la Argentina. También comparamos, mediante métodos multivariados, la composición de la micobiota con el propósito de poner a prueba dos hipótesis: 1) la micobiota está definida por las especies de hormigas, o 2) la micobiota cambia en función de la región geográfica. Se pudo observar que cada comunidad fúngica tuvo una composición y una abundancia de especies particular; las especies más frecuentes en un sitio o comunidad fue una especie rara o poco frecuente en otra comunidad o sitio. La riqueza de especies y la riqueza máxima teórica también cambiaron con los sitios. Además, encontramos que diferentes especies de hormigas en el mismo sitio tuvieron micobiotas similares, mientras que las mismas especies de hormigas en locaciones distantes tuvieron sólo unas pocas especies fúngicas en común. Por ello, concluimos que la composición de la micobiota cambia considerablemente con los sitios geográficos y que este cambio no depende de las especies de hormigas. Nuestro resultado provee nueva información sobre las hormigas cortadoras de hojas, y confirma que el jardín de estas hormigas es una comunidad fúngica muy compleja y dinámica.

[Palabras clave: abundancia, *Acromyrmex*, Ascomycota, comunidades fúngicas, riqueza, Zygomycetes]

Introduction

Attine ants (Formicidae; Myrmicinae; Attini) are a monophyletic group comprising more than 230 described species, distributed exclusively in the New World and primarily in the Neotropics (Mayhe-Nunes and Jaffe 1998; Schultz and Brady 2008; Mehdiabadi and Schultz 2010). Attine ants are divided in three groups, being one of them the leaf-cutting ants. Leaf-cutting ants depend on the cultivation of fungus for food (Höldobler and Wilson 1990). The leaf-cutting ants are represented by the genera *Acromyrmex* and *Atta*, which are the dominant herbivores of the New World tropics (Höldobler and Wilson 1990). Leaf-cutting ants provide their cultivated fungi with fresh vegetation to serve as the nutritional substrate for their fungal cultivars (Weber 1972). These ants provide the fungus with nourishment, protection from pathogens and competitors, as well as dispersion of the cultivar to new sites. New queens vertically transmit the symbiotic fungus from parent to offspring colonies (Höldobler and Wilson 1990; Currie et al. 1999).

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✉ arieljmf@hotmail.com

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All Attini fungal cultivars are asexual members of the Agaricales (Basidiomycota) (Singer 1986). Leaf-cutting ants cultivate species of *Leucoagaricus* such as *L. gongylophorus* and *L. weberi*, as well as other undefined morphotypes in the same genus (Singer 1986; Muchovej et al. 1991; Folgarait et al. 2011; Marfetán 2011; Lugo et al. 2013).

The attine fungal garden is continuously exposed to alien microorganisms (Möller 1893; Fisher et al. 1996; Rodrigues et al. 2005a,b), forming a microbiota that grow with the leaf-cutting cultivar. Microorganisms within this microbiota include: asexual fungi (Rodrigues et al. 2005a,b; Pinto-Tomás et al. 2009; Ribeiro et al. 2012), yeasts (Carreiro et al. 1997; Pagnocca et al. 2008) and bacteria (Haeder et al. 2009). Functionally, they include symbionts, saprotrophs, entomopathogens and pathogens of *Leucoagaricus* spp. Fungi growing with the cultivar or mycobiota therefore constitute a very complex community with several species growing inside the ant nest interacting in several ways.

The mycobiota of several ant species have been studied. Among them, we can find *Acromyrmex disciger* (Möller 1893), *Atta insularis* (Kreisel 1972), *Acromyrmex heyeri* (Luciano et al. 1995), *Atta cephalotes* (Fisher et al. 1996; Reis et al. 2015) and *Atta sexdens rudropilosa* (Rodrigues et al. 2005a,b). These studies revealed a considerable number of genera of Zygomycetes and asexual Ascomycetes, with species of *Mucor*, *Rhizopus*, *Cunninghamella*, *Aspergillus*, *Cladosporium*, *Escovopsis*, *Trichoderma*, *Fusarium* and *Penicillium* contributing too much of the diversity.

Despite the similarities among the mycobiotas from these different species of leaf-cutting ants, they are not identical. Although the mycobiota seems to change with the ant species, up to this point no systematic work was performed to confirm this. Moreover, the mycobiota can also change depending on the geographical site considering that it could be horizontally transmitted. In fact, *Atta cephalotes* colonies, in two different habitats from Brazil, showed unique fungal communities between the two areas (Reis et al. 2015). So far, no published studies have assessed potential changes in the mycobiota from the same *Acromyrmex* leaf-cutting ant species across geographical regions.

The present study had several goals regarding the fungal biodiversity present in leaf cutting ant nests. We describe for the first time the composition, relative frequency and the richness of the fungal species comprising the fungal community present in the nests of *Acromyrmex* species from Argentina. Additionally, we compared the composition of the mycobiota from different ant nests, from different sites and geographical regions with the purpose of testing two hypotheses: 1) the mycobiota of these ants is defined by the ant species, or 2) the mycobiota is determined by the geographical region.

Materials and Methods

We collected nests from three species of leaf-cutting ants included in the genus *Acromyrmex*: *A. lundii*, *A. lobicornis* and *A. striatus*, from nine sites that included four political provinces and

Table 1. Sampling sites, indicating GPS position, political provinces and phytogeographical regions. Additionally, number of sampled nest from each site.

Tabla 1. Sitios de muestreo. Se muestra su posición de GPS, provincia política y región fitogeográfica. Además, se presenta el número de nidos muestreados para cada sitio.

Sampling site	GPS	Province	Phyto-geographical region	Number of nests		
				A. lundii	*A. lobicornis*	*A. striatus*
La Plata	34°54′33.894′′ S - 57°56′14.28′′ W	Buenos Aires	Pampeana	8		
Berazategui	34°45′23.2446′′ S - 58°11′45.5994′′ W	Buenos Aires	Pampeana	2		
Gonnet	34°52′43.0068′′ S - 58°0′47.5194′′ W	Buenos Aires	Pampeana	5		
Santa Rosa	36°37′27.1446′′ S - 64°18′29.1594′′ W	La Pampa	Pampeana	2		4
Lihuel Calel National Park	38°0′5.76′′ S - 65°35′42.04′′ W	La Pampa	Monte		2	3
Rawson	43°19′240′′ S - 65°3′917′′ W	Chubut	Monte		5	
Puerto Madryn	42°45′57′′ S - 65°5′48.66′′ W	Chubut	Monte		4	
Luro Provincial Park	36°54′58.49′′ S - 64°15′37.62′′ W	La Pampa	Espinal			6
Las Grutas	40°48′21.09′′ S - 65°5′39.81′′ W	Río Negro	Monte			4
			Total number of nests	17	11	17

three phytogeographical regions (Pampeana, Espinal and Monte) of Argentina (Table 1 and Figure 1). Sampling was conducted between January 2011 and January 2012. Each sampling site was sampled once either in spring or summer. Differences among sampling periods were not evaluated.

Fungal isolation and maintenance

Nests were excavated as carefully as possible to ensure minimal disruption to the garden. Fungal isolations were made in the field by collecting small individual pieces of *Leucoagaricus* sp. garden material (~2 mm^3) and placing them on potato dextrose agar (PDA) with penicillin-G (100U/mL). From each nest, 20 samples were taken in situ, all from different areas of the cultivar garden. These 20 samples were considered pseudo-replicates, whereas samples from different nests of the same ant species and site were used as replicates. All the samples were taken within 5 and 8 days to the laboratory and incubated for 7-10 days at 25 °C.

Fungal identification

Pure cultures on PDA were examined using optical microscopy (Nikon, Model Eclipse E200). Fungal identification methods were based on the morphology of the fungal culture, the mechanisms of conidia production and characteristics of the conidia, following standard mycological methods (Barnett and Hunter 1998; Pitt and Hocking 2009). When identification at the species level could not be confirmed, these isolations were defined as a morphospecies and assigned with a unique code. *Escovopsis* isolates have been previously identified using two gene regions, the nuclear rDNA 28S (28S) and the TEF1 gene encoding translation elongation factor 1-α (EF-1α), and phylogenetic analyses. Amplifications were conducted with the primers: CLA-F (5'-GCATATCAATAAGCGGAGGA-3')/ CLA-R (5'-GACTCCTTGGTCCGTGTTTCA-3') and EF1-5R (5'-GTGATACCACGCTCACGCTC-3')/ EF1-3F (5'-CACGTCGACTCCGGCAAGTC-3') (Marfetán 2016)

Defining the fungal community

From each garden fragment placed in PDA, more than one taxon was found. All cultured fungi were isolated and maintained as a pure culture. Each taxon defined as a species or morphospecies was considered as a functional taxonomic unit, acknowledging that the use of morphospecies can slightly underestimate the number of species, due to the fact that morphologically cryptic isolates could have been assigned to the same morphospecies (Arnold et al. 2001).

We first compared the fungal communities present inside the nests by performing a cluster analysis to investigate the differences in the communities' composition among nests. For this analysis, an absence/presence matrix of the fungal species isolates in each nest was used. Jaccard's distance coefficient was used as the distance method and flexible beta was used as linkage method with β=-0.9 (Lance and Williams 1967).

In order to determinate if the fungal mycobiota change with ant species or with geographical site we used a two-way cluster analysis, where sites and ant species were altogether obtaining 11 sites/ant species combinations. This type of analysis also indicated which fungal species were grouped and related with the site/ant species combinations. Groups were clustered using the group average linkage method, and with the Jaccard's distance coefficient calculated for presence-absence data pertaining to fungal species.

Linkage methods used in each analysis were selected in order to avoid highly-chained dendrograms, which are undesirable as they are generally not helpful in defining subgroups (McCune and Mefford 2011). These analyses were carried out using PCOrd6 software (McCune and Mefford 2011).

Structure of the fungal communities

Once the scale of the community was defined in the previous step we proceeded to describe them by a) calculating species accumulation curves in order to evaluate whether the sampling effort was sufficient, and to calculate the theoretical maximum richness, and b) evaluating species abundance. To calculate species accumulation curves, we used EstimateS software (Colwell 2005), and the curves were smoothed using the rarefaction method (Moreno 2001; Gotelli and Colwell 2001), after which they were adjusted using the Clench method to obtain the theoretical maximum richness for each fungal community (Moreno 2001). Data from different pseudo-replicates were considered only once per nest from the same species/mophospecies. Finally, the species abundance was calculated as the number of times a particular species

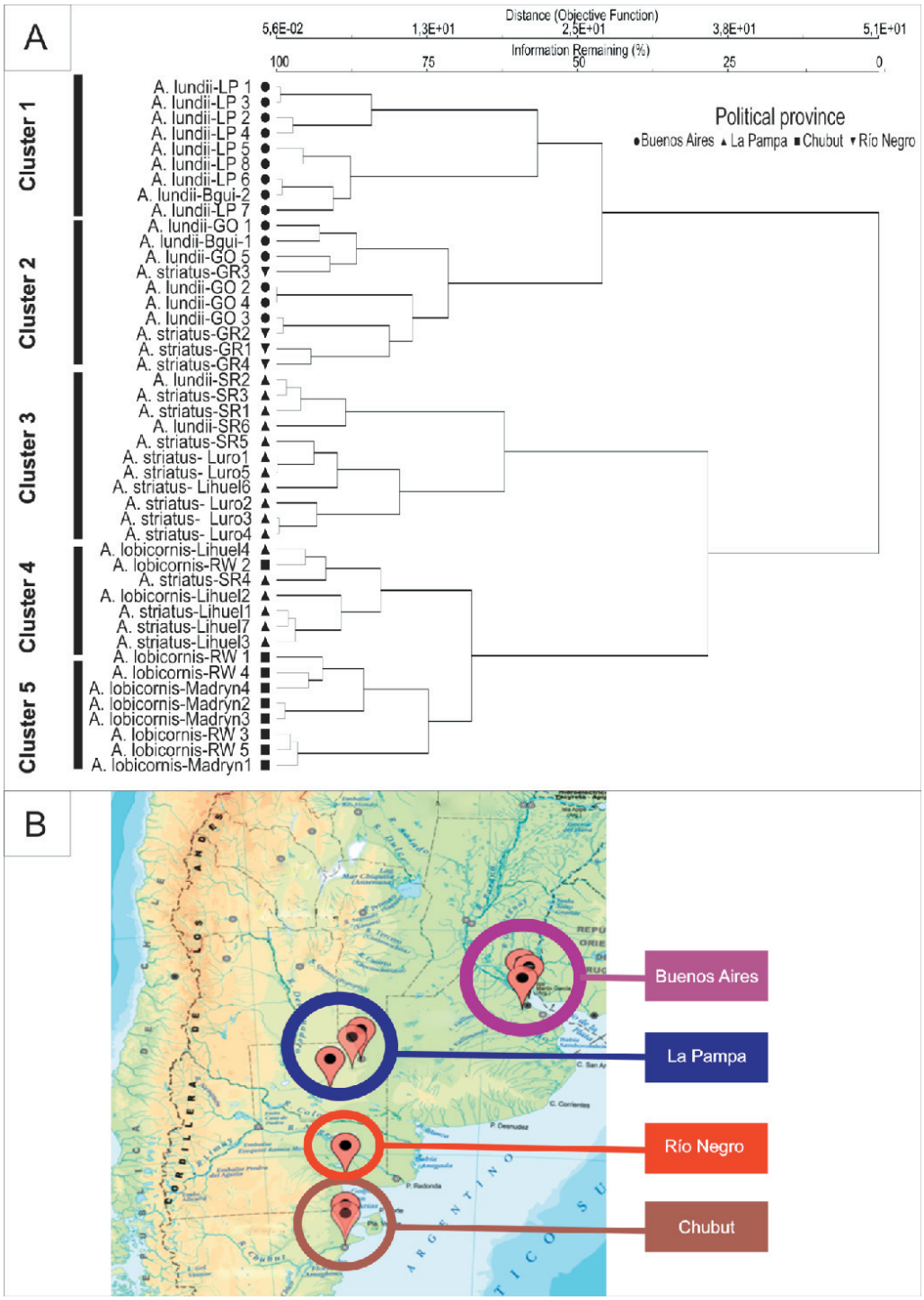

Figure 1. A) Cluster analysis grouping ant nests based on the composition of mycobiota. Symbols represent nests from different geographic provinces. Site's names were codified as: Bgui=Berazategui, GO=Gonnet, GR=Las Grutas, Lihuel=Lihuel Calel National Park, LP=La Plata, Luro=Luro Provincial Park, Madryn=Puerto Madryn, RW=Rawson, SR=Santa Rosa. B) Partial map of Argentina. Circles show the four different fungal communities grouped by political province.

Figura 1. A) Análisis de grupos juntando nidos de hormigas en función de la composición de la micobiota. Los símbolos representan nidos de diferentes provincias. Los nombres de los sitios de muestreo fueron codificados de la siguiente manera: Bgui=Berazategui, GO=Gonnet, GR=Las Grutas, Lihuel=Parque Nacional Lihuel Calel, LP=La Plata, Luro=Parque Provincial Luro, Madryn=Puerto Madryn, RW=Rawson, SR=Santa Rosa. B) Mapa parcial de la Argentina. Los círculos muestran las cuatro comunidades fúngicas diferentes agrupadas por provincias.

or morphospecies was observed divided by the total number of isolates. As mentioned previously, each isolate from the same species obtained from the same nest was considered as a pseudo-replicate. All pseudo-replicates were counted as 1 regardless of repetitions, in order to maintain the nests as independent sampling units.

RESULTS

Fungal identification and fungal community definition

We obtained 605 fungal isolates, 544 of which were Ascomycetes and 61 Zygomycetes. Within these isolates, we identified 39 Ascomycetes taxa (species and morphospecies) included in 21 genera, as well as 12 Zygomycetes taxa, belonging to 9 genera.

In the dendrogram obtained from cluster analysis, we defined five clusters (Figure 1). Cluster 1 and 2 grouped samples from Buenos Aires and showed more similarity between them versus the other clusters. Samples from Las Grutas (Río Negro province) were grouped with samples from one site of Buenos Aires. Cluster 3 comprised three subclusters. All subclusters were formed by nests from La Pampa province. Cluster 3 was more similar to cluster 4 and 5. Cluster 4 was almost exclusively formed by nests from Lihuel Calel Park. Cluster 5 grouped all nests from the two sample sites in Chubut province. This analysis showed that nests from the same province, were grouped together (with few exceptions), showing that fungal communities from the same sampling site were very similar. Moreover, cluster 1 and 2 grouped samples from Buenos Aires whereas 3 and 4 joined functional taxonomic units from La Pampa, whereas the fifth grouped samples from Chubut.

The two-way cluster analysis defined four clusters for the site/ant species combinations. The first one was formed by *A. lundii* nests isolated from three sites in Buenos Aires province. The second cluster comprised the nests of *A. striatus,* which was the only species present at Las Grutas. Again, the mycobiota of these nests were highly similar to the mycobiota found in nests from Buenos Aires. The third group included all of the ant species sampled in the three sites from La Pampa province: *A. lundii*, *A. striatus* and *A. lobicornis*. Finally, the fourth cluster grouped the *A. lobicornis* nests sampled in Chubut province (Figure 2).

Fungal communities were clearly defined by closeness among geographical sites where the samples were taken, but not by the leaf-cutting ant species. For this reason, we defined the communities as a function of the geographical site. Communities were named as Buenos Aires, La Pampa, Chubut and Río Negro, referring to the geographical province where samples were taken from (Figure 1b). The two-way cluster analysis showed that although several fungal species were shared across sites, each site had its own set of unique species (2-A). We found taxa such as *Absidia* sp., *Cunninghamella echinulata var. nodosa*, *Gibberella fujikuroi*, *F. proliferatum*, *Stigmella* sp., *Escovopsis weberi* and *Zigorhyncus* sp. only in nests from Buenos Aires. Similarly, species such as *Cunninghamella septata, Phialophora* sp., *Acrospeira* sp., *Ampellomyces* sp., *Doratomyces* sp., *Metarhizium anisopliae, Mucor* sp., *Nigrospora* sp. and *Scopulariopsis* sp. were very frequently present in all sites from La Pampa province, but were absent in the other geographical regions or provinces.

The fungal communities from Chubut province had their own non-shared taxa, such as *Mortierella* sp., *Thermomucor* sp., *Penicillium implicatum* and *Penicillium rugulosum*. Moreover, *Beauveria bassiana* was another species that, despite being cosmopolitan was only isolated from Chubut nests. Finally, we found in Río Negro only one endemic taxa, *Cunninghamella echinulata* var. *nodosa* (Figure 2a).

Structure of fungal communities

Table 2 describes the initial number of isolates and the final number of species/ morphospecies, as well as singletons found in each community. Using the number of taxa found in the above-defined communities, we constructed species accumulation curves as a function of the number of leaf-cutting ant nests sampled to describe their maximum theoretical richness and evaluated how well these communities were sampled.

We found that La Pampa was the community with the highest theoretical maximum richness (44 species), followed by Buenos Aires and Río Negro (36 and 35 species, respectively) and Chubut, which had the lowest theoretical maximum richness (31 species) (Figure 3). Taking into account the richness found empirically (Table 2), our sampling captured 77.27% of the richness from La Pampa, 66.66% of the possible taxa from Buenos Aires, and 61.71% from Chubut. In Río Negro, our

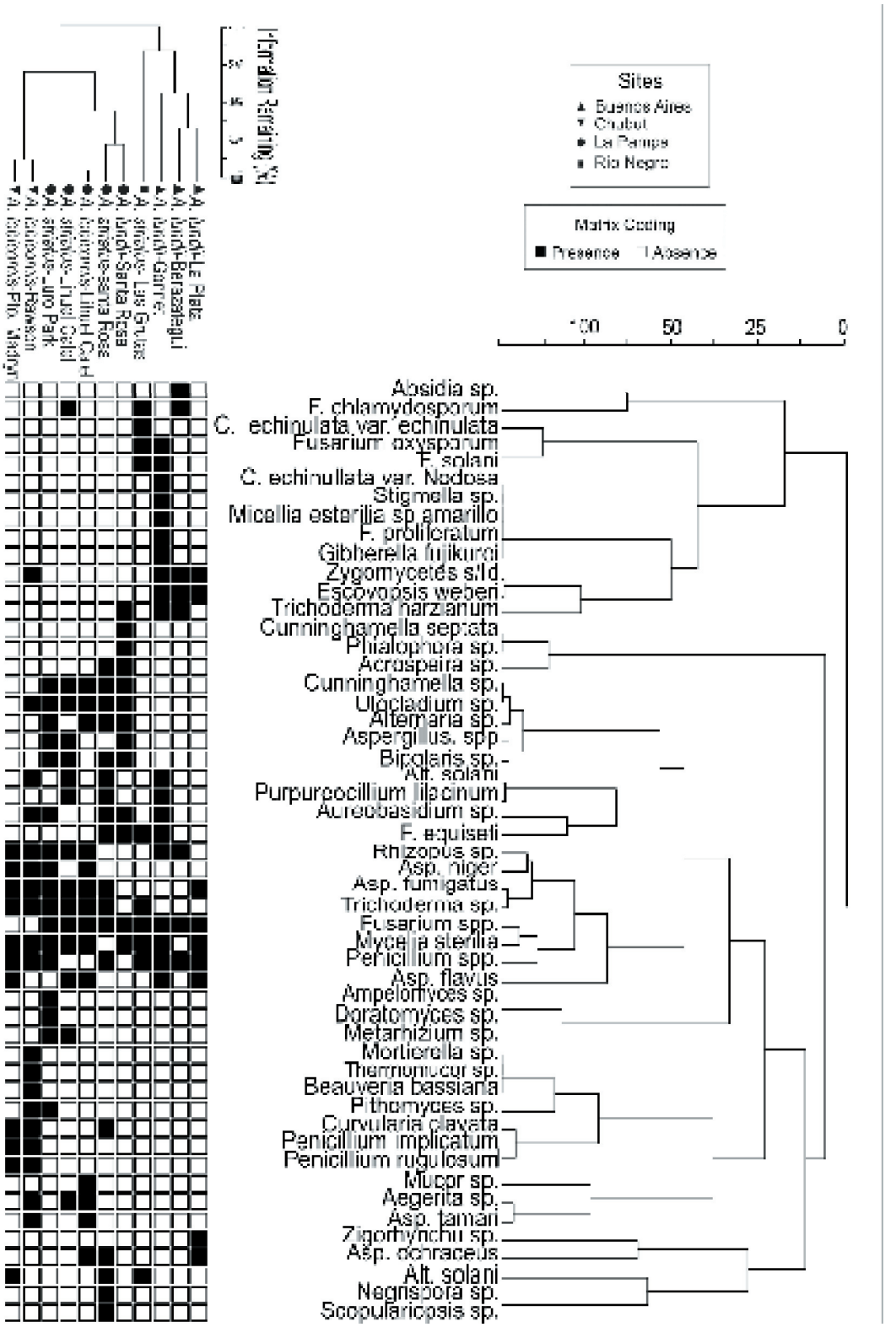

Figure 2. Two-way cluster analysis grouping fungal taxa by sites/ant species combination.

Figura 2. Análisis de grupos de dos vías agrupando los taxones fúngicos en función de las especies de hormigas separadas por sitios.

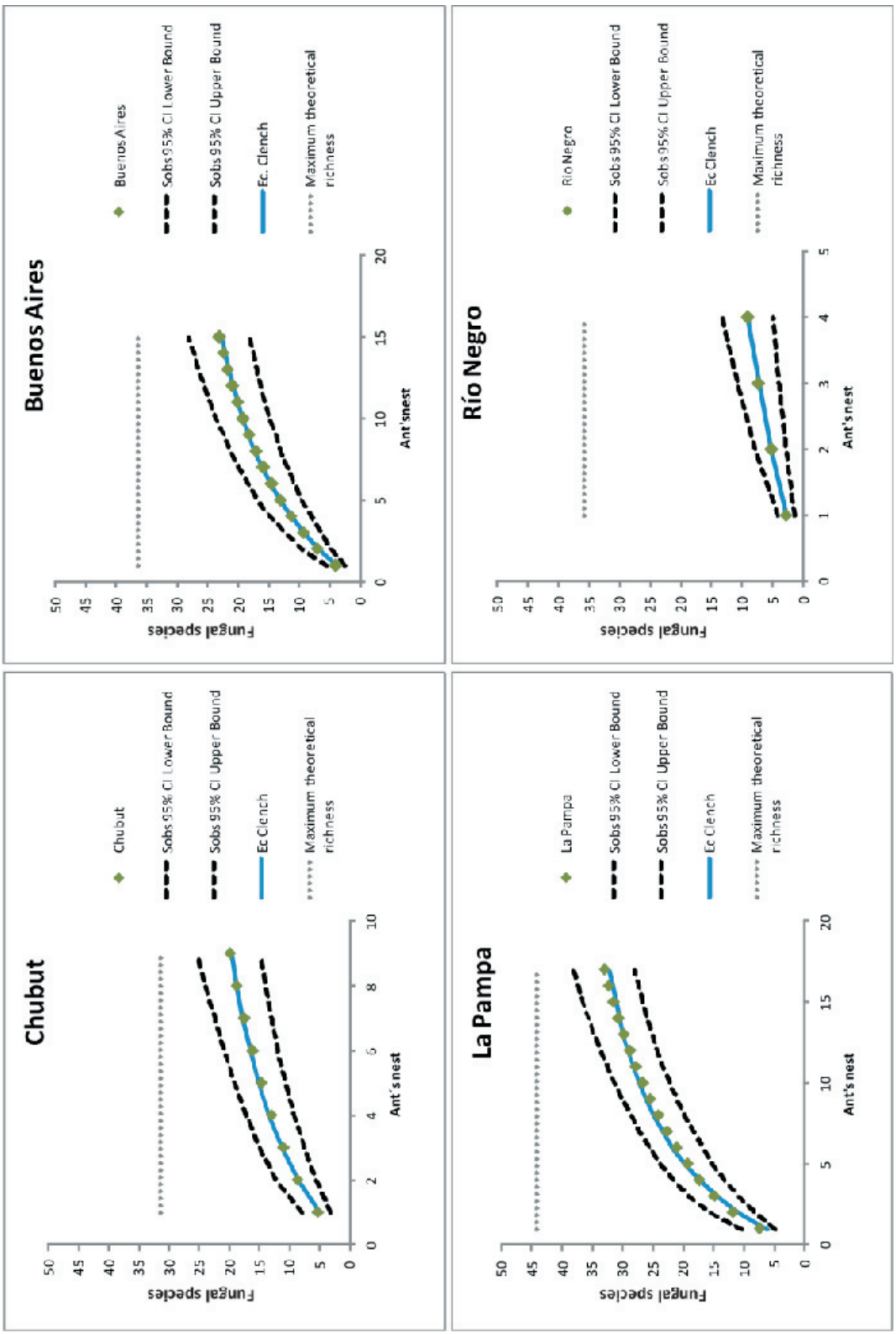

Figure 3. Species-accumulation curves showing the relationship between the total number of fungal species found and the number of nests sampled at each community.

Figura 3. Curvas de acumulación de especies mostrando la relación entre el número total de especies encontradas y el número de nidos muestreados para cada comunidad.

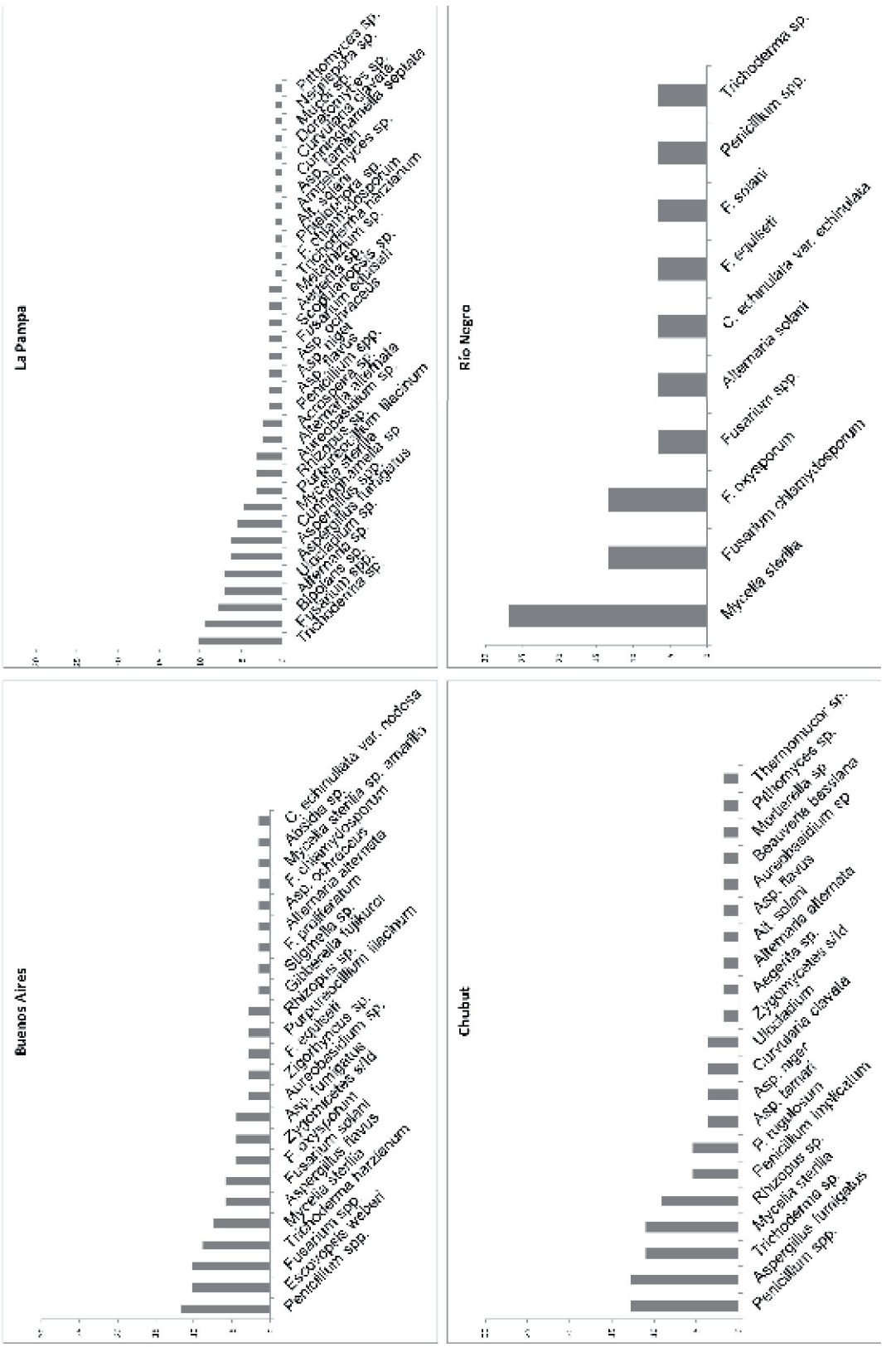

Figure 4. Abundance of fungi by communities
Figura 4. Abundancia de hongos en cada una de las comunidades.

Table 2. Total number of isolates and genera discriminated in Ascomycetes and Zygomycetes. Additionally, richness and singletons are shown per site.

Tabla 2. Número total de aislamientos y de géneros separados en Ascomycetes y Zygomycetes. Además, se muestran la riqueza y los singletons hallados en cada sitio.

	La Pampa	Buenos Aires	Chubut	Río Negro	Total
Isolates	282	167	129	27	605
Ascomycetes isolates	258	150	111	25	544
Ascomycetes genera	20	8	10	5	21
Ascomycetes taxa	30	18	17	9	74
Zygomycetes isolates	24	17	18	2	61
Zygomycetes genera	3	5	4	1	9
Zygomycetes taxa	4	6	4	1	12
Richness (n° species and morphospecies)	34	24	21	10	51
Singletons	9	6	9	4	-

sampling was clearly insufficient, obtaining only 28.57% of the theoretical richness.

In Buenos Aires the most frequent taxa were *Penicillium* spp. (11.59%), *E. weberi* (10.14% of isolates), *Fusarium* spp. (10.14%) and *Trichoderma harzianum* (8.69%). Additionally, we found a large number of isolates that did not produce reproductive structures, denominated as *mycelia sterilia* (7.24%). However, the mycobiota of leaf cutting ants in La Pampa had a very different composition. The most frequent taxa was *Trichoderma* sp. (10.15%), followed by *Fusarium* spp. (9.37%), *Bipolaris* sp. (7.81%), *Alternaria* sp. (7.03%), *Ulocladium* sp. (7.03%), *Aspergillus* spp. (6.25%), *Aspergillus fumigatus* (6.23%) and *Cunninghamella* sp. (5.46%). The *mycelia sterilia* were also very frequent in this region (4.68%). There were 24.5% of species (13 species) in common between both provinces (Figure 4).

The richness of the mycobiota from Chubut was similar to that from the other sites, but the taxa composition and frequency was different. In Chubut, the most frequent species/ morphotypes were *Penicillium* spp. (12.5%), *Aspergillus fumigatus* (12.5%), *Trichoderma* sp. (10.71%), *mycelia sterilia* (10.71%), *Rizopus* sp. (8.92%), *Penicillium implicatum* (5.35%) and *Penicillium rugulosum* (5.35%). Chubut had more species in common with La Pampa (28.8%; 15 species), than with Buenos Aires (15.4%; eight species) and Río Negro (7.7%; four species). The most frequent taxa in Río Negro were *F. chlamydosporum* (13.3%), *mycelia sterilia* (26.6%) and *F. oxysporum* (13.3%) (Figure 4). The mycobiota of this province had more species in common (11.5%; six species) with La Pampa than with Buenos Aires (9.6%, five species) or Chubut (7.7%, four species).

Finally, in all communities we found singletons, there were six singletons in Buenos Aires (*Alt. alternata, Asp. ochraceus, F. chlamydosporum, Micellia esterilia* sp., *Absidia* sp. *and Cunninghamella echinullata var. Nodosa*), nine singletons in La Pampa (*Alt. solani, Ampelomyces* sp., *Asp. tamari, Cunninghamella septata, Curvularia clavata, Doratomyces* sp., *Mucor* sp., *Negrispora* sp., and *Pithomyces* sp.), nine singletons in Chubut (*Aegerita* sp., *Alt. alternata, Alt. solani, Asp. flavus, Aureobasidium* sp., *Beauveria bassiana, Mortierella* sp., *Pithomyces* sp. and *Thermomucor* sp.) and four in Río Negro (*F. equiseti, F. solani, Penicillium* spp. and *Trichoderma* sp.) (Figure 4).

Discussion

This study described the biodiversity of culturable fungi in the gardens of several species of *Acromyrmex* leaf-cutting ants across different geographical sites, political provinces and phytogeographical regions. We found 21 genera of Ascomycetes and nine genera of Zygomycetes, which allowed us to define the species composition of the mycobiotas as well as the differences across the fungal communities and the most frequent fungal species in each case. Our most important result was that the fungal composition of the gardens was not dependent on the ant species. The composition of the mycobiota changed considerably with geographical site. Different species of ants from the same site had similar mycobiota, while the same ant species in distant sites had few fungal species in common. This suggests a locality effect in the diversity of the mycobiota rather than an effect caused by ant nest architecture or sanitary behaviours. The species richness, as well as the theoretical maximum richness, also changed with sites.

Previous studies concluded that soil communities of microorganism, such as

fungal and bacterial communities, can vary in richness and diversity across ecosystem types and geographical regions (Hawkins et al. 2003; Fierer and Jackson 2006; Lauber et al. 2008). These changes through different sites within the same region can be explained by environmental factors such as soil pH (Fierer and Jackson 2006), mean annual temperature and potential evapotranspiration (Hawkins et al. 2003). Other factors that can be key regulators of the biogeographical patterns exhibited by fungal communities are soil texture, total C, total N and extractable P (Lauber et al. 2008). The plant diversity at each site can also play a very important role in the frequency of the transient mycobiota found in ant nests. The leaf material cut by attine ants normally contains a great number of endophytic and epiphytic fungi (Fisher et al. 1996; Fröhlich and Hyde 1999; Hashizume et al. 2008; Suryanarayanan et al. 2009). Fisher et al. (1996) proved that changes in the plant substrate cut by ants can lead to changes in the fungal community found in *Atta cephalotes* nests due to the endophytic species present in the plant. This fact could explain the diversity pattern found across sites in our study. Some highly common fungi found in this study such as *Alternaria alternata*, *Curvularia* spp., *Fusarium* spp., *Trichoderma* spp. and *Aureobasidium* sp. grow in plants as endophytes. Other fungi such as *Aspergillus* spp., *Bipolaris* sp., *Penicillium* spp. and *Trichoderma* spp. can grow in plants, some of them as parasitic organisms (Fisher et al. 1996; Fröhlich and Hyde 1999; Hashizume et al. 2008; Shali et al. 2010; Suryanarayanan et al. 2009; Vázquez de Aldana et al. 2013). The functional role of the mycobiota present in leaf-cutting ant colonies is still not known. However, some endophytic species from the vegetation that ants carry into their colonies could be pathogens of the ant´s cultivar. Among the endophytic species that could be opportunistic parasites we can find species in the genus *Trichoderma* (Rocha et al. 2017), and previous work showed that leaves with species of *Fusarium*, and *Ulocladium* are rejected by ants, probably because some of these genera include mycoparasites and could represent a threat to the fungal gardens (Rocha et al. 2014). Additionally, it is well known that species in the genus *Escovopsis* are specialist pathogens of the leaf-cutting ants cultivar (Currie et al. 1999, 2003). In this work we also found species that have been reported as entomopathogenic fungi. *Beauveria bassiana* and *Metarhizium* sp. are two of the most studied pathogens of these ants (Jaccoud et al. 1999; Castilho et al. 2010), but species in the genus *Fusarium* and *Aspergillus* have been also reported as possible causes of queen mortality in leaf-cutting ant nest (Marti et al. 2015). For the rest of the species their possible functional role remains unknown.

In this study, we analysed if fungal communities changed with phytogeographical regions. Most large scale environmental factors and vegetation patterns are summarized within the concept of phytogeographical regions. Lihuel Calel Park (La Pampa), Las Grutas (Río Negro), Rawson and Pto. Madryn (Chubut) are in the Monte region, whereas Buenos Aires and Santa Rosa belong to the Pampeana region, and Parque Luro (La Pampa) is in the Espinal region (Cabrera 1976). The mycobiota proved to be very different among sites, even though they shared the same phytogeographical region. Las Grutas, with all samples from Chubut and Lihuel Calel park, and Santa Rosa and all sites in Buenos Aires are in the same phytogeographical region, but their mycobiotas were very different. Therefore, the phytogeographical regions are not good predictors of the fungal communities found in garden of leaf-cutting ants, probably due to the fact that differences in fungal communities could be related with local environmental factors.

When the frequency of the fungal taxa was assessed, changes across geographical sites were also evident. We found that species highly frequent in a site can be absent or at a minimal frequency in another. An example of this is the case were *Bipolaris* sp. and *Penicillium* spp., which were the most frequent genera in La Pampa and Chubut province, respectively, but were almost absent in the other regions. Once again, environmental factors can account for this kind of change.

Escovopsis weberi was one of the most frequently-occurring taxon in nests from Buenos Aires, but was absent in the other regions. This was particularly unexpected considering that other authors described *Escovopsis* species as the most frequent parasite in the fungal chambers (Currie et al. 1999, 2003; Currie and Stuart 2001). In parasitized nests, *E. weberi* was one of the most frequent species, but this symbiotic species was absent from several sites. In La Pampa, sampling was carried out in summer (December) and *Escovopsis* species were not found. However, in subsequent sampling in spring (October) *E. weberi* was found. Similarly, in a second

sampling in Buenos Aires we found three more *Escovopsis* morphotypes. These results could suggest that season could affect the presence of the *Escovopsis* species and this result is in accordance with previous works where the mycobiota changed seasonally (Rodrigues et al. 2011).

In the present work, we used a cultured-depended approach, and it is possible that the culture media used in this work (PDA) could have underestimated the biodiversity of the mycobiotas. This media tends to recover fast-growing fungal species that are better adapted to assimilate glucose, being the obtained biodiversity skewed to such group of fungi. Biodiversity could be also underestimated due to the fact that several isolates were defined as morphospecies and considered as a functional taxonomic unit. The use of morphospecies can slightly underestimate the number of species (Arnold et al. 2001) because more than one taxon could be group in the same morphospecies. This underestimation could be most important in the *mycelia sterilia* group, where all isolates that did not produce conidia or conidiophores were joined together even knowing that comprise more than one unidentified species. Further analyses using more sensitive techniques will be needed and could reveal additional fungal taxa and new information, showing a higher biodiversity in the mycobiota present in the ants´ colonies. In spite of the limitations of this technique, our results provide a new understanding of the leaf cutting-ants system, proving that the mycobiota present within the gardens are independent of the ant taxa or phytogeographical province but highly dependent on the geographical sites were nests are located. These results agree with the notion that the fungi found in the garden are horizontally transmitted and suggest non co-evolution with the cultivar.

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