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Comparative analysis of fungal communities in colonies of two leaf-cutting ant species with different substratum preferences

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ABSTRACT

Fungus gardens of leaf-cutting ants harbor diverse alien fungi in addition to their fungal cultivar. Previous work suggested that alien microorganisms are likely derived from the substrata foraged by ant workers and incorporated into the fungus gardens. To test this hypothesis, we sampled 1014 garden fragments from 16 field colonies of *Atta sexdens rubropilosa* (a dicot-cutting ant) and *Atta capiguara* (a grass-cutting ant) in Brazil. From a total of 615 fungal isolates recovered, we observed similar diversity of fungi between colonies of both ant species. However, fungal communities differed in composition of taxa between ant colonies. *Trichoderma spirale*, *Trichosporon chiarellii* and *Penicillium citrinum* were prevalent accounting for 18.5%, 12.2% and 11.7% of the total isolates, respectively. As expected, fungal communities clustered in two major groups supporting the hypothesis that plant substratum has an impact on the composition of the alien fungi found in leaf-cutting ant gardens.

1. Introduction

Leaf-cutting ants are a paramount example of interactions between insects and multiple microbial symbionts. They belong to the tribe Attini (Hymenoptera: Formicidae) which comprises ants that cultivate fungi for food. Depending on the species, ant workers collect either fresh dicot or monocotyledonous leaves as substratum to nourish the mutualistic fungus, *Leucoagaricus gongylophorus* (Basidiomycota: Agaricaceae). In turn, the fungus is the main food source for the brood (Weber, 1972; Silva et al., 2003). Workers accumulate the foraged plant material in the fungus garden, a sponge-like structure composed of fungal mycelium and plant substratum carefully tended by the ants (Weber, 1972). Due to their leaf-cutting habit, these ants are considered pests in agricultural areas causing major economic losses, especially in South America (Della Lucia et al., 2014).

The fungus garden harbors a complex microbiome in addition to the resident fungal mutualist, including filamentous fungi, yeasts and bacteria (Möller, 1893; Fisher et al., 1996; Carreiro et al., 1997; Currie et al., 1999a; Rodrigues et al., 2008; Suen et al., 2010; Montoya et al., 2016). Microbes found in this substratum may be commensals (transients) or may act as (i) auxiliary-microbes in the garden enzymatic metabolism (Suen et al., 2010; Ayward et al., 2012), (ii) as disease-suppressing organisms (Rodrigues et al., 2009; Ishak et al., 2011) or (iii) as pathogens such as the specialized fungal parasite *Escovopsis* (Currie et al., 1999a). Except for the latter fungus which is only found in association with attine ant gardens, most microbes present in the fungus garden are likely derived from the soil adjacent to ant colonies, from the integuments of workers and alates (Little and Currie, 2007; Arcuri et al., 2014; Atilli-Angelis et al., 2014) or from the plant substratum used for nourishing the fungal mutualist (Van Bael et al., 2009, 2012a; Coblentz and Van Bael 2013).

Despite the significant effort dedicated to characterize the microbiome in the fungus garden, little is known about the factors that determine the structure of garden microbial communities. Rodrigues et al. (2011) reported changes in the dynamics of

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filamentous fungal communities in ant gardens over a year-long survey suggesting that the type of substratum may explain variations in fungal diversity. However, the influence of different substrata in alien fungal communities (i.e. fungi not cultured by the ants) of attine gardens has not been systematically studied to date.

Leaf-cutting ant species exhibit diverse preferences for plant substrata to cultivate their fungi. For example, *Atta sexdens rubropilosa*, the most widespread leaf-cutter ant in Brazil, cuts leaves from dicotyledonous plants (Fowler et al., 1986; Andrade et al., 2002). On the other hand, *Atta capiguara* and *Atta bisphaerica* are typical grass-cutting ants mostly found in pastures and grasslands (Fowler et al., 1986; Garcia, 2005). Regardless of the type of substratum, workers process the plant material which decreases the alien fungal loads and the diversity of fungi before incorporating it into the fungus gardens (Andrade et al., 2002; Van Bael et al., 2009, 2012b; Diniz and Bueno, 2010). Exploring the differences between ant species with distinct substratum preference is useful to shed light on whether fungi other than *L. gongylophorus* are either transients or resident components of attine ant gardens. To assess the influence of substratum preferences in fungal communities, we evaluated the dynamics of such fungi in gardens of *A. capiguara* and *A. sexdens rubropilosa* in two consecutive years.

2. Materials and methods

2.1. Fungus garden sampling

Colonies of *A. capiguara* (a grass-cutting ant) and *A. sexdens rubropilosa* (a dicot-cutting ant) were located on a farm in the municipality of Botucatu, São Paulo state, Brazil (Table S1). At this site *A. capiguara* cuts grasses in a field used to rear cattle, whereas *A. sexdens rubropilosa* nests and forages adjacent to the pasture within stands of *Eucalyptus* trees.

Four colonies of *A. capiguara* and four colonies of *A. sexdens rubropilosa* were excavated in April 2012, and four additional colonies of each ant species were sampled in January and March 2013 ($n = 16$ colonies sampled in total, Table S1). Because these two species have fungus gardens enclosed in underground chambers, colonies were carefully excavated following Rodrigues et al. (2009). Based on field observations *A. sexdens rubropilosa* colonies were apparently more than 4 years-old and *A. capiguara* colonies were more than 2 years-old (except for colony N10 that had the same age then as *A. sexdens* colonies). All ant colonies had multiple garden chambers.

Fungus gardens from the top chambers of each colony, along with tending ants and brood, were collected and kept in UV-sterilized plastic containers with a thin layer of plaster in the bottom which was moistened with 4 ml of sterile distilled water. Samples were transported to the laboratory and kept at 25 °C for up to 4 d after collecting (Table S1). Over this time, no leaves were provided to the ants, and the exhausted fungus garden parts separated by the ants were removed from the containers with a sterile spatula. Ant foragers and minor workers were collected and stored in 96% alcohol as vouchers, and are kept at the Center for the Study of Social Insects (UNESP, Rio Claro).

2.2. Fungal isolation and identification

To check the prevalence of alien fungi in each sample, garden fragments (approximately 3 mm^3) without workers or brood were inoculated on three different culture media: potato dextrose agar (PDA, Acumedia), 2% malt agar (MA2% Acumedia) and synthetic nutrient agar (SNA), all supplemented with 150 $\mu g\ ml^{-1}$ of chloramphenicol (Sigma). A total of 25 garden fragments were inoculated on five Petri plates (five fragments per plate) in each of the three culture media. Thus, a total of 75 garden pieces were plated for each of the 16 samples, totaling 1200 garden pieces examined in the study. The garden pieces were randomly removed from the top and bottom parts of the fungus gardens, which represent the fresh and old garden parts, respectively. Plates were incubated at 25 °C up to 14 d and observed daily for fungal growth. Once a fungus was detected in a garden piece, mycelium fragments (or spores in the case of sporulating fungi) were transferred to a MA2% plate. Then the corresponding garden piece from the isolation plate was removed with an ethanol-flamed spatula and discarded, to prevent overgrowth by fast-growing fungi.

Fungi subcultured in MA2% were initially grouped into morphospecies according to colony morphology and microscopic characteristics of reproductive structures. The latter were observed in wet-mounts and compared to those available in taxonomic keys (Barron, 1968; Carmichael et al., 1980; Domsch et al., 1980; Pitt, 2000; Samson et al., 2000). Fungi that did not sporulate were inoculated on oatmeal agar (OA) containing sterile banana leaves and incubated at 25 °C in the dark up to 2 months. After this procedure, those fungi which did not sporulate were considered sterile mycelia. Representative isolates are kept at UNESP - Microbial Resources Center (CRM - UNESP) in 10% glycerol at - 80 °C, and are available upon request.

The internal transcribed spacer (ITS) region of the rDNA gene was amplified using primers ITS4 and ITS5 (White et al., 1990) for representative isolates from each morphospecies. PCR conditions followed Rodrigues et al. (2014) and amplicons were cleaned up with the Wizard® SV Gel and PCR Clean-Up System kit (Promega). Purified amplicons were quantified with a NanoDrop® (Thermo Scientific) and 20 ng of DNA was prepared for sequencing using BigDye Terminator® v. 3.1 Kit (Life Technologies) according to the manufacturer's protocol. Forward and reverse sequences were generated using the same primers on an ABI 3330xl (Life Technologies) and compiled into contigs in BioEdit v. 7.0.5.3 (Hall, 1999). Sequences generated in the present study were deposited in the NCBI-GenBank database under accession numbers: **KR093827 – KR093967**.

Contigs were queried for homologous sequences at the NCBI-GenBank and the CBS (www.cbs.knaw.nl) databases. Sequences that showed over 97% identity with those deposited in the databases were considered if they were consistent with morphology (Unterseher and Schnittler, 2010; see also Table S3). For isolates belonging to the genus *Trichoderma* we used the *Trich*OKEY barcode database to find the best matches (Druzhinina et al., 2005).

To further ensure correct taxonomic affiliation of fungi, phylogenetic trees were inferred using homologous sequences retrieved from the databases. Sequences were aligned using MAFFT v. 7.110 (Katoh and Standley, 2013). The trees were generated in MEGA v. 5.2 (Tamura et al., 2011), using the neighbor-joining algorithm, Kimura 2-parameter as nucleotide substitution model and 1000 bootstrap pseudoreplicates. Such phylogenies were generated for each major group of fungi found in the present study and are available upon request.

2.3. Analyses of fungal communities

The prevalence of fungi in gardens of *A. capiguara* and *A. sexdens rubropilosa* was compared using the proportion of garden fragments with alien fungi. A test for equality of proportions was used to compare the proportions of garden fragments positive for alien fungi between ant species and between culture media. This comparison was carried out in R v. 3.0.1 (R Development Core Team, 2013). In addition, rarefaction curves were built for comparison between communities (Colwell, 2013), and the estimated richness of fungal taxa was calculated using the Chao 1 (Magurran and Gill,