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Absence of the Parasite *Escovopsis* in Fungus Garden Pellets Carried by Gynes of *Atta sexdens*

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Abstract

Before the mating flight, the gyne of leaf-cutting ants takes a small pellet of their mutualistic fungus garden to start fungus culture in its new colony by vertically transmitting it. This mutualism is threatened by the specialized microfungal parasite *Escovopsis*, which is exclusively associated with the ant's fungus gardens. Evidences suggest that *Escovopsis* transmission between colonies is horizontal, i.e. the parasite is transferred between established nests. However, such studies analyzed a relatively small number of fungal pellets or were restricted to a few ant colonies. Additionally, there is a report of rapid parasite dispersion, compatible with a winged vectored mechanism, suggesting that there is also vertical transmission. Herein, we carried out a complementary study on the possibility of vertical transmission of *Escovopsis* by sampling a large number of fungus pellets from gynes of *Atta sexdens*, a species not previously studied from this perspective. Gynes were collected during their mating flights in 2009 and 2010, and were left in moist chambers upon fungus regurgitation. Each pellet was inoculated on potato dextrose agar and incubated at 25 °C, resulting in prevalence of the mutualistic cultivar, low proportions of other fungal species, and absence of *Escovopsis*. Thus, our study consolidates the results of previous reports that *Escovopsis* vertical transmission does not occur or is negligible, thus enabling the characterization of this parasite transmission as horizontal. Future studies on *Escovopsis* transmission mechanisms may explain why, although horizontal, it seems to be as fast as the transmission mediated by winged vectors.

Introduction

Leaf-cutting ants in the genera *Atta* Fabricius and *Acromyrmex* Mayr (Hymenoptera: Formicidae) cultivate a mutualistic fungus for food. The ants maintain the fungal cultivar (*Leucoagaricus gongylophorus* (Moller) Singer; Basidiomycota: Agaricales) in a structure named the fungus garden, composed of fungal mycelium and plant material collected by workers. Fungus gardens are threatened by the specialized microfungal parasite *Escovopsis* Muchovej & Della Lucia (Ascomycota: Hypocreales), which negatively impacts the development of the colony. This parasite is highly virulent and may overgrow the ant gardens leading to the death of the colony (Currie et al., 1999; Currie, 2001a). It is suggested that microorganisms, especially antibiotic-secreting actinobacteria found on the integument of workers protect the fungus gardens against this parasite (Currie, 2001b; Mueller et al., 2008; Caldera et al., 2009; Barke et al., 2011).

Before leaving for the mating flight gynes collect a small fungus garden fragment and stores it in the infrabuccal pocket, which is located in the oral cavity of the ant. Then, males and gynes from different leaf-cutting ant colonies simultaneously leave their parental nest to mate during the mating flight. Once fertilized gyne reach the ground, they remove their wings and start the foundation of a new colony. The fungal pellet is then regurgitated by the gyne who starts the culturing of the new incipient fungus garden. This mode of transmission of the fungal cultivar is considered vertical (from parental to offspring colony). In *Atta*, the gyne is responsible for the excavation of a tunnel and the first nest chamber in which she remains underground for about two months (claustral nest founding). After this period, the first emerged workers reopen the tunnel that connects to the ground surface (Autuori, 1941; Moser, 1967).

Much is known about the interaction between leaf-cutting ants and their mutualistic fungus, however, little

is known about *Escovopsis* biology, especially its mode of transmission (Currie, 2001b; Yek et al., 2012). Two studies, Currie et al. (1999) and Pagnocca et al. (2008) evaluated the transmission of the parasite between colonies. These reports indicate that no vertical transmission appears to occur (Table 1). Currie et al. (1999) evaluated pellets of gynes in their mating flight and in the fungus gardens of colonies in the claustral phase. However, the sampling size was relatively small and the study was restricted to one ant species (*Atta colombica* Guérin-Méneville in Panama). On the other hand, a second study evaluated the integument and fungal pellets of gynes collected immediately before the mating flight (when gynes were leaving the parental colony). Although, Pagnocca et al. (2008) used a larger sampling size in comparison to the Currie et al.'s (1999) study (Table 1) only one colony of *Atta laevigata* F. Smith and two colonies of *Atta capiguara* Gonçalves were sampled, in Brazil. In addition, the variation of *Escovopsis* prevalence between ant species and locations are aspects that prompt for additional studies to extend the existing data on the mode of transmission of this parasite (Currie, 2001a; Yek et al., 2012).

Considering that *Escovopsis* was not found in air samples collected next to the ant colonies, and because its spores are typically slimy when mature (wet spores), it is inferred that it is horizontally transmitted (between established nests) through the migration of commensal arthropods, or via some other unknown mechanism (Currie, 2001b; Poulsen & Currie, 2006; Yek et al., 2012). Moreover, multiple lines of evidence indicate this parasite appears to have a close relationship with fungus growing ant colonies because: (i) it was only found in the fungus gardens and waste material (Muchovej & Della Lucia, 1990; Seifert et al., 1995; Currie et al., 1999; Rodrigues et al., 2014); (ii) phylogenetic studies showed that specific *Escovopsis* lineages are specialized on specific lineages of the ant cultivar (Currie et al., 2003; Gerardo et al., 2006a,b); and (iii) additional energy is necessary to grow the actinobacteria which are considered as a defense mechanism against this parasite, and the cultivation of such actinobacteria is enhanced in response to *Escovopsis* infection (Poulsen et al., 2003).

Interestingly, Mikheyev (2008) demonstrated that a recent colonization by *Acromyrmex octospinosus* Reich in the Guadeloupe Island was probably initiated by a single fertilized gyne or colony derived from the continent. Surprisingly, *Escovopsis* was found in 12% of the established colonies on the island and worker ants were covered with white blooms of actinobacteria on their integuments. In addition, a study on young colonies of *Atta colombica* (Currie et al., 1999) indicates that shortly after the reopening of the tunnel, the fungus gardens of these colonies were infected by *Escovopsis*. Therefore, it may be considered that (i) the mechanisms of horizontal transmission of the parasite are quick and efficient, or (ii) vertical transmission is possible.

Table 1. Studies involving the isolation of filamentous fungi from leaf-cutting ants during the mating flight period.

Study	N	Sample type	Ant species	Year of collection	Site
Currie et al. (1999)	38	Gyne pellets, after the mating flight*	*Atta colombica*	1998	Gamboa, Panama
	8	Gardens of incipient laboratory colonies** (aseptic rearing)	*Atta colombica*	1998	Gamboa, Panama
	22	Gardens of incipient field colonies** (in claustral phase)	*Atta colombica*	1998	Gamboa, Panama
Pagnocca et al. (2008)	144	Gyne pellets + cuticle, before the mating flight*	*Atta laevigata* (1 colony)	2006 + 2007	Santana farm, Botucatu, Brazil
	120	Gyne pellets + cuticle, before the mating flight*	*Atta capiguara* (2 colonies)	2006 + 2007	
This work	267	Gyne pellets, after the mating flight*	*Atta sexdens*	2009 + 2010	Lageado farm, Botucatu, Brazil

* Isolation attempts were carried out in the same day of the mating flight.

** Age of few weeks.

Because *Escovopsis* is a specialized parasite (Currie et al., 1999, 2003; Poulsen & Currie, 2006; Yek et al., 2012), it can hypothetically exploit the fastest and most efficient mode of transmission, i.e. vertical transmission. This hypothesis may be considered plausible because it explains the result of Mikheyev's (2008). Similarly, in systems of figs and their symbionts, the specific phytonematode *Schistonchus* Cobb is transmitted rapidly and efficiently by its winged vector (fig wasp, pollinator of figs): this transmission is vertical, permitting a colonization of new figs upon the arrival of this pollinator (Vovlas et al., 1992; Krishnan et al., 2010).

Thus, an additional investigation of the presence of *Escovopsis* in fungal pellet, the most likely location for this parasite to be present in case of vertical transmission (Currie et al., 1999), is critical. In the present work we investigate if *Escovopsis* utilizes the mode of vertical transmission by carrying out a larger survey of *Atta sexdens* Linnaeus gyne pellets and comparing our results to existing studies. We focused our work on *Atta sexdens* because it is one of the most common and

widespread leaf-cutting ant species in Latin America (Solomon et al., 2008), and due to the fact that the gynes of this species have not been investigated for the presence of *Escovopsis*.

Material and Methods

For gyne collection, we selected a small site (c.a. 1.3 ha) in a *Eucalyptus* spp. plantation at Lageado Farm (22°49'53.25" S and 48º25'24.22" W), in Botucatu, SP, Brazil.

This area did not contain any mature *Atta* nests, but it was being used as an intense landing site of fertilized gynes. *Atta* gynes can fly up to several km in nuptial flights (Moser, 1967; Jutsum & Quinlan, 1978; NS Nagamoto, personal obs.); thus, gynes collected in our small area actually came from surrounding areas (fragments of native forest, and plantation areas for such crops as cereals, grasses, *Eucalyptus* L'Heritier, and *Citrus* Linnaeus). In undisturbed forests, the *Atta* spp. colony density is about 0.23 ha^{-1}, lower than the trend in anthropically disturbed areas (Wirth et al., 2007 and references included); thus, potentially dozens or hundreds of mature *A. sexdens* nests had contributed gynes to the collection area.

This neighborhood includes some study areas where *Escovopsis* was previously found: in a small colony of Rodrigues et al. (2014) and in another work (NS Nagamoto et al., unpublished data), it was demonstrated that one of the colonies studied by Pagnocca et al. (2008), denominated "*Atta capiguara* colony 1", was indeed infected with this parasite. Additionally, based on the long persistence of these ants' colonization in these areas (NS Nagamoto, personal obs.), it was inferred that the rate of colonies infected by *Escovopsis* was probably high.

Gynes were sampled in two consecutive years: (i) on October 21th, 2009, 147 gynes were collected, of which 127 pellets were used, and (ii) on October 31th, 2010, 150 gynes, of which 140 pellets were used (some gynes did not regurgitate pellets; a few pellets were lost to contamination by bacteria; or no fungus grew on pellets). Sampling was carried out after the mating flight, when gynes were still walking on the soil, or just after they started the excavation of the first tunnel. Ants were collected with tweezers and placed into sterile disposable Petri dishes that were wrapped in plastic bags.

In the laboratory, gynes were transferred to a wet chamber (Fig 1A), which consisted of a Petri dish of 60 x 12 mm, that was placed in a bottom of a larger Petri dish of 100 x 16 mm, with addition of 20 mL of water agar (as a source of moisture) between the plates, and covered by the 100 mm Petri plate cover; and then, sealed with PVC film. After 24 hours, the regurgitated pellets were aseptically inoculated in Petri dishes containing Potato Dextrose Agar (PDA, Acumedia) supplemented with 50 mg L^{-1} of penicillin G and streptomycin sulfate (Sigma) according to Currie *et al.* (1999). Plates were sealed with PVC film and incubated at 25 °C in the dark. Plates were visually inspected over 21 days and once a fungus was detected on the pellets, we subcultured it in PDA in order to obtain pure cultures. Filamentous fungi identification was performed using morphological markers as stated in the classical taxonomic keys (Domsch et al., 1980; Samson et al., 2000). Specific literature was used for morphological identification of the mutualistic fungus (Pagnocca et al., 2001) and *Escovopsis* (Muchovej & Della Lucia, 1990; Seifert et al., 1995).

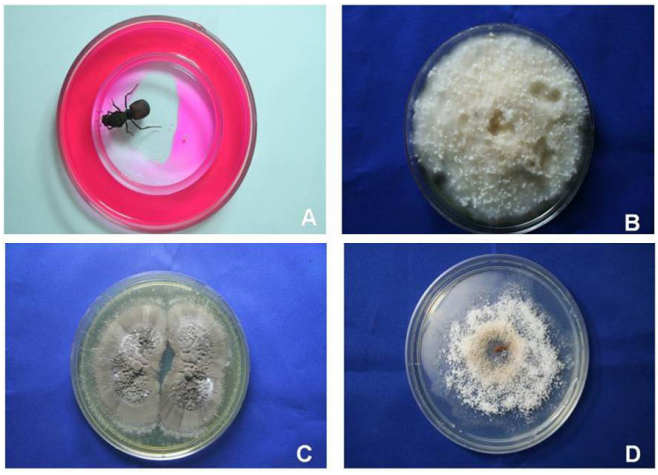

Fig 1. General aspect of the wet chamber used in the study. Moist chambers were made from Petri dish of 60 x 12 submerged in an another dish of 100 x 16 mm containing water-agar (medium dyed red for contrast for picture) to promote regurgitation of pellets of the mutualistic fungus (A); the mutualistic fungus (B); filamentous fungi isolated from the pellet (C); parasitic fungus *Escovopsis*, isolated from a colony of *Atta sexdens* (D). For B, C and D, the culture medium is PDA (Potato Dextrose Agar).

Results and Discussion

In accord with previous studies of other *Atta* species (Currie et al., 1999; Pagnocca et al., 2008), our research supports the hypothesis that *Escovopsis* (Fig 1D) is not found in the pellets of *Atta sexdens* gynes (Fig 2). As expected, *L. gongylophorus* (Fig 1B) was recovered in a high proportion of the pellets collected in the two year sampling (70.1% in 2009 and 85.0% in 2010). In addition, other filamentous fungi (Fig 1C) were isolated from pellets but at lower proportions (29.9% in 2009 and 15.0% in 2010) when compared to the mutualistic fungus.

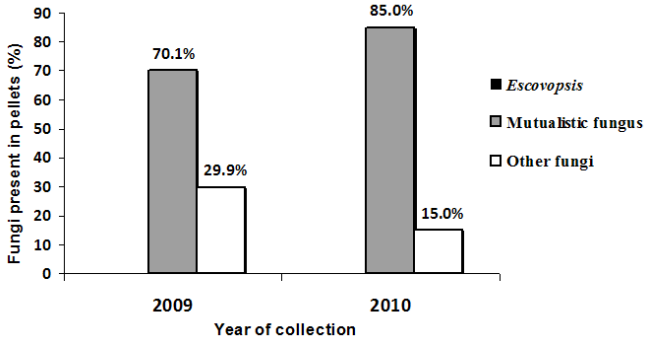

Fig 2. Prevalence of filamentous fungi in pellets extracted from gynes of *Atta sexdens* collected in the mating flights of 2009 (n = 127 pellets) and 2010 (n = 140). The specialized parasite fungus *Escovopsis* was not found in the survey.

Pagnocca et al. (2008) also found a significant presence of other filamentous fungi in the gyne's infrabuccal pellets, indicating these microorganisms may be accidentally acquired together with the fungal garden fragments. In both studies, the number of pellets where the fungal mutualist was recovered was higher than the number of pellets with the presence of non-mutualistic fungi. Many fungi such as *Cladosporium* Link found in pellets of *A. capiguara* and *A. laevigata* (Pagnocca et al., 2008) are fast growing, and may be found in lower proportions in the fungal pellets when compared with the mutualistic fungus. However, such fungi still can easily overgrow the ant cultivar in the absence of the ants' care but may not impact the successful initiation of a new garden by the gyne (Poulsen & Currie, 2006).

Our results corroborate those of previous studies, thus indicating that vertical transmission of *Escovopsis* does not exist or, if it exists, is negligible. Both horizontal and vertical transmission can be used by some specific parasites to infect a host population (Fries & Camazine, 2001). However, as to parasites that possess specific mechanisms such as chemotaxis , vertical transmission is more plausible: in fig systems, the plant parasitic nematode *Schistonchus* presents chemotaxis to its vector (fig wasp, pollinator of figs) and thus accomplishes its vertical transmission by this mechanism (Krishnan et al., 2010). Thus, this example contrasts with the non-vertical transmission of *Escovopsis* because it also presents chemotaxis, in this case, to the mutualistic fungus of leaf-cutting ants (Gerardo et al., 2006a), and since this mutualist is vertically transmitted by winged gynes.

Horizontal transmission is expected in parasites that are generalist and widespread in the environment, such as *Metarhizium* Sorokin, which causes only sporadic horizontal infection in leaf-cutting ant workers (Hughes et al. 2004). In contrast, taking into account that *Escovopsis* quickly pursued the *Acromyrmex* dispersion in islands (Mikheyev, 2008), has shown fine evolutionary coupling to the mutualistic fungus (Gerardo et al., 2006b), presents chemotaxis (Gerardo et al., 2006a), is found only in fungus growing ant nests (Currie et al., 1999, 2001a,b; Rodrigues et al., 2014) and in their dumps (Currie et al. 2001a), and is not transmitted by air (Currie et al., 1999, 2001b) – non-vertical transmission was not the most expected result. Additionally, the bacterium *Paenibacillus larvae* White, that causes the specific disease American Foulbrood in *Apis* Linnaeus bee larvae, is transmitted vertically and horizontally (Fries & Camazine, 2001), in contrast to the lack of vertical transmission of *Escovopsis*.

Therefore, for now, the mode of *Escovopsis* transmission may be better explained by emphasizing some specific mechanisms, rather than the general patterns of parasite transmission. Leaf-cutting ant workers can detect fungal pathogens, such as *Escovopsis*, when performing their grooming behavior (Currie & Stuart, 2001; Yek et al., 2012); therefore, it is likely that gynes can also achieve this detection when gathering material to form their infrabuccal pellet. Thus, gynes may be capable of selectively exploring the healthiest portions of fungus garden, focusing on the upper half of the garden, where *Escovopsis* is less likely to be found (Currie, 2001a). If these explanations are correct, even if *Escovopsis* is present in a colony, this parasite would not be transported by gynes when they leave for their nuptial flight. This may explain why the present work and other studies (Currie et al., 1999; Pagnocca et al., 2008) have not detected *Escovopsis* in fungal pellets.

Thus, in contrast with the low efficiency of horizontal transmission of non-specialized parasites, such as *Metarhizium*, which are widely distributed in the environment (Hughes et al. 2004), it is indicated that there may be highly effective specific mechanisms for horizontal transfer of *Escovopsis*, which is only found in colonies of fungus-growing ants. Future studies that investigate the horizontal transmission of *Escovopsis* should clarify why the phenomenon appears to occur as rapidly as transmission mediated by winged vectors, and specify the mechanisms that augment transmission efficiency.

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