Fungi Isolated from Wild Birds and Litter in the Itatiaia National Park in Southeastern Brazil

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ABSTRACT – In Brazil, the Atlantic Forest has suffered from deforestation, which has caused impacts on its flora, fauna, and microbiota. This biome is considered one of the main tourist and birdwatching destinations due to the large number of species, especially Passeriformes. However, the fungal diversity present in these environments is little known and studied. In this work, a total of 148 samples from 74 wild birds (74 feathers and 74 feces) and 16 samples of litter were collected in Itatiaia National Park, southeastern Brazil. Filamentous fungi isolated from these samples were identified using macroscopic and microscopic characteristics. Among birds, *Aspergillus* spp., *Mucor* spp., *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp. and *Syncephalastrum* spp. were identified in higher abundance. In litter, *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. were identified. These results indicate the presence of saprophytic fungi species in the feathers and feces of wild birds and in the litter of the capture site. Further studies should be conducted in order to elucidate if the mycobiota profile modifies with anthropization and if it interferes with bird health and environmental recovery.

Keywords: Passerines; microbiota; environment.

Fungos Isolados de Aves Silvestres e Serrapilheira no Parque Nacional do Itatiaia no Sudeste do Brasil

RESUMO – No Brasil, a Mata Atlântica tem sofrido com desmatamento, que vem causando impactos na sua flora, fauna e microbiota. Esse bioma é considerado um dos principais destinos turísticos e de observação de aves, devido ao grande número de espécies, sobretudo Passeriformes. Entretanto, a diversidade fúngica presente nesses ambientes é pouco estudada e conhecida. Neste trabalho, 148 amostras de 74 aves silvestres (74 de penas e 74 de fezes) e 16 amostras de serapilheira foram coletadas no Parque Nacional do Itatiaia, no sudeste brasileiro. Fungos filamentosos isolados dessas amostras foram identificados utilizando características macroscópicas e microscópicas. Entre as aves, *Aspergillus* spp., *Mucor* spp., *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp. e *Syncephalastrum* spp. foram identificados em maior abundância. Em serapilheira, *Aspergillus* spp., *Fusarium* spp. e *Penicillium* spp. foram identificados. Esses resultados indicam a presença de espécies de fungos saprófitas nas penas e nas fezes de aves silvestres e na serapilheira do local de captura. Mais estudos devem ser realizados a fim de elucidar modificações no perfil da micobiota com a antropização e sua interferência na saúde das aves e recuperação ambiental.

Palavras-chave: Passeriformes; microbiota; meio ambiente.

Hongos Aislados de Aves Silvestres y Arpillera en el Parque Nacional de Itatiaia en el Sureste de Brasil

RESUMEN – En Brasil, la Mata Atlántica ha sufrido por la deforestación, lo que ha causado impactos en su flora, fauna y microbiota. Este bioma es considerado uno de los principales destinos turísticos y de observación de aves debido a la gran cantidad de especies, principalmente Passeriformes. Sin embargo, la diversidad fúngica presente en estos ambientes es poco conocida y estudiada. En este

trabajo, se recolectaron un total de 148 muestras de 74 aves silvestres (74 plumas y 74 heces) y 16 muestras de hojarasca en el Parque Nacional Itatiaia, en el sureste de Brasil. Los hongos filamentosos aislados de estas muestras se identificaron utilizando características macroscópicas y microscópicas. Entre las aves, *Aspergillus* spp., *Mucor* spp., *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp. y *Syncephalastrum* spp., se identificaron en mayor abundancia. En la hojarasca, *Aspergillus* spp., *Fusarium* spp. y *Penicillium* spp. fueron identificados. Estos resultados indican la presencia de especies de hongos saprofitos en las plumas y heces de las aves silvestres y en la hojarasca del sitio de captura. Se deben realizar más estudios para dilucidar los cambios en el perfil de la micobiota con la antropización y su interferencia en la salud de las aves y la recuperación ambiental.

Palabras clave: Paseriformes; microbiota; ambiente.

Introduction

The Atlantic Forest is a Brazilian moist tropical forest, considered one of the greatest biodiversity hotspots, however, it has been suffering from deforestation, which has been causing impacts on its flora and fauna (Rosa *et al.*, 2018). In Rio de Janeiro, the Itatiaia National Park was the first protected area created in Brazil and has 28,155 hectares of remaining forest in Serra da Mantiqueira (Rosa *et al.*, 2018). The Park is considered one of the main tourist and birdwatching destinations in Brazil due to the local species richness (Berto & Lopes, 2020). Birds are extremely important for the maintenance of this ecosystem, as they play an important role in plant pollination, seed dispersal, pest control, among others (Berto & Lopes, 2020). On the other hand, birds are among the animals that can function as a reservoir and a disperser for various agents that cause zoonoses, among these microorganisms are fungi, which can be associated with feathers, when they bump into some substrate, and found in internal organs, entering through the feeding paths (Simi *et al.*, 2019).

The wild birds of the Atlantic Forest suffer from the stress caused by deforestation and anthropization, becoming more susceptible to infection of various types of microorganisms (Cordeiro *et al.*, 2021). Colonization by opportunistic fungi can be facilitated by morphological and physiological characters that favor their development, including poorly vascularized air sacs and defect uropygial glands with difficulty producing secretions to be distributed through the plumage by means of preening (Berto & Lopes, 2020). The fungi, after associating with their host, if not eliminated by the cells of the immune system, enter a commensal or parasitic relationship with it, which can cause infection. This process depends on the general health status of the host (immunocompromised hosts are susceptible) and on the inherent characteristics of the microorganism, as efficient virulence factors (Casadevall & Pirofski, 2000; Feitosa *et al.*, 2020).

The vast majority of environmental fungi are saprophytes, commonly or occasionally found in soil, decomposing vegetation, seeds, and grains. Many of these are opportunistic, i.e., in contact with immunocompromised hosts they can cause disease (Pitt, 1994). Some of these species have been recognized as important pathogens in humans or immunocompromised domestic animals (Pitt, 1994; Simi *et al.*, 2019; Arné *et al.*, 2021). However, as the fungal diversity present in the tropical environment, most of it still needs to be discovered and understood; it is possible and common that fungi in the environment are capable of parasitizing animals (Nardoni & Mancianti, 2021; Arné *et al.*, 2021). This explains the importance of establishing the occurrence and frequency of fungi in the environment and in potential hosts.

Passeriformes birds living in wild environments are carriers and dispersers of fungi in nature (Della Vedova *et al.*, 2019; Nardoni & Mancianti, 2021). This dispersion in the environment can occur mostly in the following ways: through the release of their feces in the environment, and through contact with their body parts, as these fungi can be associated with the on feathers (Warner & French, 1970; Simi *et al.*, 2019; Kraisitudomsook *et al.*, 2021). Therefore, it is important to establish the profile of fungi found in feathers and feces (birds) and litter (environment) and to establish the incidence of total filamentous fungi. In this context, this work sought to identify

filamentous fungi isolated from samples of wild birds and litter, in order to understand the diversity and frequency of fungal species within bird habitats in the Itatiaia National Park, southeastern Brazil.

Material and Methods

Study site and sample collection

This study was carried out along the Travessia Ruy Braga in Itatiaia National Park, a protected area with a high degree of vulnerability, located in the Mantiqueira Mountains, Southeastern Brazil (22°26'17 "S; 44°37'33 "W). The expeditions were carried out in May, June and July 2021. The captures took place 3 days a month, and 10 mist nets were used, totaling 180 meters, and they remained open from 5 am to 5 pm, totaling 12 hours day and 36 hours a month. A total of 74 birds of different species were captured (Table 1). Birds were kept in individual boxes and feces were collected immediately after defecation and packed in sterilized centrifuge tubes. The birds were identified according to Pacheco *et al*. (2021). The feathers (plumage and tail) were removed with sterile tweezers and placed in previously sterilized white paper envelopes to eliminate moisture, thus preventing the growth of contaminating fungi and/or bacteria. After obtaining the samples the birds were released in the same environment where they were captured. Additionally, a total of 19 samples of litter were collected every 500 meters from the beginning of the Travessia Ruy Braga in a course of 6.5km in the lower part of the Itatiaia National Park. All samples were properly labeled, packed in thermal bags at room temperature and transported to the Laboratory of Mycology and Mycotoxicology of the Federal Rural University of Rio de Janeiro.

Fungal isolation

Five to 10mg of feces were streaked on Sabouraud agar (Difco) plus chloramphenicol and each sample was incubated directly in a Petri dish (90 x 15cm) at 28°C for up to 07 days (Simi *et al*., 2019).

Whole and clipped feathers were streaked on Mycosel® Agar (Difco) and each sample was incubated in a Petri dish at 28°C for up to 07 days (Nardoni & Mancianti, 2021).

The litter samples were placed in Petri dishes, in which sterilized horsehair threads and distilled water (both sterile) were deposited. The samples were incubated at 25°C for up to four weeks following the methodology of Vanbreuseghem (1952).

For the identification of the fungi grown on the plates and on the manes, the following characteristics were observed: growth characteristics of the colonies, such as color and appearance (macromorphology) and characteristics of mycelium, presence, shape, size and septation of macroconidia; abundance and roughness of microconidia; presence or absence of chlamydoconium; presence or absence of forms of sexual reproduction; hyphal septation (Samsom *et al*., 2000; Sidrim *et al*., 2004; De Hoog *et al*., 2021).

Results

A total of 148 samples from 74 wild birds captured were collected and included feathers (n=74) and feces (n=74) (Table 1), as well as 16 litter samples. From each sample it was possible to isolate one filamentous colony or more; therefore, the number of fungi isolated does not correspond to the total number of samples. Out of a total of 117 fungi isolated, 17 genera were identified, as shown in Table 2. The genera that occurred in greater numbers were *Aspergillus* and *Mucor* followed by *Cladosporium*, *Fusarium*, *Penicillium*, and *Syncephalastrum*. A total of 22 morphospecies could be identified (18%), among them *Aspergillus* section *Nigri*, *Aspergillus* section *Circumdati*, *Aspergillus* section *Fumigati* and *Penicillium citrinum*.

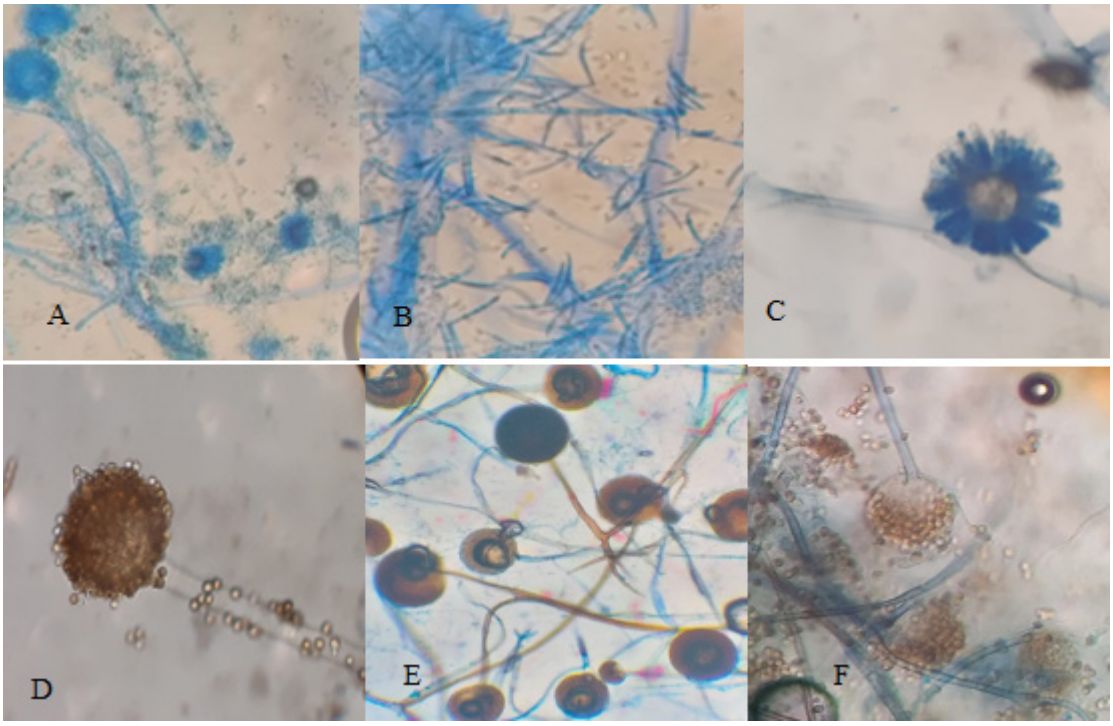

Figure 1 – Light microscopy of (A) *Aspergillus* sp. with lactophenol cotton blue; (B) Fusarium sp. with lactophenol cotton blue; (C) *Syncephalastrum* sp. with lactophenol cotton blue; (D) *Aspergillus* sp. with sodium hydroxide (20%); (E) *Rhizopus* sp. with lactophenol cotton blue; (F) *Mucor* sp. with lactophenol cotton blue.

Table 1 – Bird species (Pacheco *et al*., 2021), samples, and isolated fungi.

Family	Bird species	Feathers	Feces
Thraupidae	*Cissopis leverianus* (Gmelin, 1788)	*Mucor* sp.	*Geotrichum* sp., *Aspergillus* section *Nigri*
	Saltator similis (d'Orbigny & Lafresnaye, 1837)	*Mucor* sp. *Fusarium* sp.	*Aspergillus* section *Fumigati*
	Saltator fuliginosus (Daudin, 1800)		
	Trichothraupis melanops (Vieillot, 1818)	*Aspergillus* sp., *Aspergillus* section *Nigri*	*Eurotium* sp.
	Tachyphonus coronatus (Vieillot, 1818)	*Aspergillus* section *Nigri*	
	Tachyphonus coronatus	*Aspergillus* section *Nigri*	
	Tachyphonus coronatus	*Aspergillus* section *Nigri*	
	Tachyphonus coronatus	*Aspergillus* section *Nigri*	
	Saltator fuliginosus	*Mucor* sp.	*Aspergillus* section *Nigri*
	Trichothraupis melanops	*Mucor* sp.	*Aspergillus* section *Nigri*
	Tachyphonus coronatus	*Mucor* sp.	*Eurotium* sp.
	Haplospiza unicolor (Cabanis, 1851)	*Mucor* sp. *Fusarium* sp.	*Aspergillus* section *Fumigati*
	Stephanophorus diadematus (Temminck, 1823)		*Aspergillus* section *Nigri*
	Stephanophorus diadematus	*Lichtheimia* sp.	*Fusarium* sp.
	Stephanophorus diadematus		*Cladosporium* sp.
	Saltator similis		*Aspergillus* section *Circumdati*, *Cladosporium* sp.
	Stephanophorus diadematus	*Alternaria* sp.	
	Stephanophorus diadematus		*Cladosporium* sp.
	Saltator similis		*Cladosporium sp.*, *Fusarium* sp.
	Stephanophorus diadematus	*Mucor* sp.	
	Thachyphonus coronatus	*Chrysonilia* sp.	
	Stephanophorus diadematus	*Acremonium* sp.	*Cladosporium* sp.
	Tangara sayaca (Linnaeus, 1766)		*Aspergillus* section *Nigri*
	Tangara desmarestis (Vieillot, 1819)	*Penicillium* sp.	*Fusarium* sp.
	Tangara desmarestis		*Syncephalastrum* sp.
	Tangara desmaristis		*Curvularia* sp., *Fusarium* sp.
	Stephanophorus diadematus	*Cladosporium* sp.	
	Microspingus lateralis (Nordmann, 1835)		*Rhizopus* sp.
	Saltator similis		*Rhizopus* sp.
	Trichothraupis melanops		*Rhizopus* sp.
	Microspingus lateralis (Nordmann, 1835)		*Rhizopus* sp.
	Tangara desmarestis		*Syncephalastrum* sp.
	Trichothraupis melanops		
	Microspingus lateralis	*Mucor* sp.	*Cladosporium* sp., *Mucor* sp., *Fusarium* sp.

Parulidae	*Chiroxiphia caudata* (Shaw & Nodder, 1793)		
	Manacus manacus (Linnaeus, 1766)	*Mucor* sp.	
	Chiroxiphia caudata		*Cladosporium* sp.
	Neopelma chrysolophum (Pinto, 1944)	*Chaetomium* sp.	
	Myiothlypis leucoblephara (Vieillot, 1817)		*Cladosporium* sp.
	Myiothlypis leucoblephara	*Mucor* sp.	*Aspergillus* section *Flavi, Cladosporium* sp., *Mucor* sp.
	Basileuterus culicivorus (Deppe, 1830)		*Syncephalastrum* sp., *Mucor* sp.
	Basileuterus culicivorus		*Mucor* sp.
	Basileuterus culicivorus		
	Myiothlypis leucoblephara		
	Myiothlypis leucoblephara	*Curvularia* sp., *Mucor* sp.	*Syncephalastrum* sp.
Onychorhynchidae	*Myiobius atricaudus* (Lawrence, 1863)		*Aspergillus* section *Fumigati*
	Myiobius atricaudus		*Neosartorya* sp.
Thamnophilidae	*Thamnophilus caerulescens* (Vieillot, 1816)	*Lichtheimia* sp.	
	Thamnophilus caerulescens		*Aspergillus* section *Flavi, Mucor* sp.
	Thamnophilus caerulenscens		*Penicillium* sp.
	Pyriglena leucoptera (Vieillot, 1816)		
	Pyriglena leucoptera	*Neosartorya* sp.	*Aspergillus* section *Fumigati*
	Pyriglena leucoptera	*Cladosporium* sp., *Penicillium* sp.	
Furnariidae	*Anabazenops fuscus* (Vieillot, 1816)		*Aspergillus* section *Fumigati*
	Anabacerthia amaurotis (Temminck, 1823)	*Penicillium* sp.	
	Cranioleuca pallida (Wied, 1831)		*Fusarium* sp., *Cladosporium* sp.
Passerellidae	*Zonotrichia capensis* (Statius Muller, 1776)	*Mucor* sp.	*Curvularia* sp., *Chaetomium* sp.
	Zonotrichia capensis	*Mucor* sp.	
	Zonotrichia capensis	*Lichtheimia* sp.	
	Phylloscartes difficilis (Ihering & Ihering, 1907)		*Mucor* sp.
Rhychocyclidae	*Phylloscartes difficilis*		*Fusarium* sp.
	Phylloscartes difficilis		*Mucor* sp.
	Phylloscartes difficilis		*Curvularia* sp.
Vireonidae	*Hylophilus poicilotis* (Temminck, 1822)	*Penicillium* sp.	
	Hylophilus poicilotis		*Bipolaris* sp.
	Hylophilus poicilotis	*Penicillium* sp.	*Syncephalastrum* sp.
Tyrannidae	*Knipolegus cyanirostris* (Vieillot, 1818)		*Cladosporium* sp., *Mucor* sp.
	Knipolegus cyanirostris	*Mucor* sp.	*Fusarium* sp.
Fringillidae	*Euphonia pectoralis* (Latham, 1801)	*Neosartorya* sp.	*Eurotium* sp.

Conopophagidae	*Conopophaga melanops* (Vieillot, 1818)		*Aspergillus* section *Fumigati*, *Aspergillus* section *Nigri*
Dendrocolaptidae	*Sittasomus griseicapillus* (Vieillot, 1818)	*Lichtheimia* sp.	
Turdidae	*Turdus flavipes* (Vieillot, 1818)		*Aspergillus* section *Fumigati*
Tityridae	*Schiffornis virescens* (Lafresnaye, 1838)	*Fusarium* sp.	*Neosartorya* sp.
Plathyrinchidae	*Platyrinchus mystaceus* (Vieillot, 1818)		

Table 2 – Frequency list of fungi identified in the three substrates analyzed.

Fungi	Feathers	Feces	Litter
Acremonium spp.	1		
Alternaria spp.	1		
Aspergillus spp.	6	17	2
Bipolaris spp.		1	
Chaetomium spp.	1	1	
Chrysonilia spp.	1		
Cladosporium spp.	2	11	
Curvularia spp.	1	3	
Eurotium spp.		3	
Fusarium spp.	3	8	5
Geotrichum spp.		1	
Lichtheimia spp.	4		
Mucor spp.	14	8	
Neosartorya spp.	2	2	
Penicillium spp.	5	1	5
Rhizopus spp.		4	
Syncephalastrum spp.		5	
Total	40	65	12

Discussion

Most of the species identified in this work can be considered environmental and opportunistic fungi (depending on the general condition of the host), however, the pathogenicity of the fungi was not evaluated in this study. The most abundant in the sampled areas were, *Aspergillus* spp. and *Mucor* spp. followed by *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp. and *Syncephalastrum* spp.

Similar results were found by Bills & Polishook (1994), when studying the abundance and diversity of fungi from Costa Rican moist forest litter, they found *Penicillium* spp., *Trichoderma* spp., *Paecilomyces* spp., *Aspergillus* spp., and Mucorales fungi, the latter two in larger quantities, as in this study. Research also in the Atlantic Forest of southeastern Brazil conducted by Bezerra *et al*. (2020), in the Restinga de Jurubatiba National Park in Rio de Janeiro,

reported equivalent results, being isolated *Aspergillus* spp., *Penicillium* spp., *Curvularia* spp., *Pestalotiopsis* spp., *Bipolaris* spp., *Monilia* spp., *Nigrospora* spp., and *Trichoderma* spp.

It is noteworthy that studies involving the mycobiota present in wild birds, mainly Passeriformes, despite their great importance, are rare and, in the Itatiaia National Park, no survey of the mycobiota present in the feathers or feces of these animals has been done so far. This is the first study to report saprophytic fungi in wild birds in Itatiaia National Park.

The identification of the genera *Aspergillus* and *Penicillium* in migratory birds are carried out frequently, e.g., Akter *et al*. (2020) isolated *Aspergillus* spp. and *Penicillium* spp. in feces of migratory birds in Bangladesh. Simi *et al*. (2019) identified *Aspergillus* spp. in feces of parrots and birds of prey in captivity in central-western Brazil. Among the fungi identified in the present study in Itatiaia National Park, we highlight *Aspergillus* spp., which occurred abundantly in feces. This genus can be found in soil, organic matter, and many other places. Aspergillosis, a disease caused by *Aspergillus* sp., can affect the respiratory tract of immunocompromised birds in general, and cause severe pneumonia, being one of the main causes of death in captive birds and, less frequently in free-living birds (Joseph, 2003, Talbot *et al*. 2018; Della Vedova *et al*., 2019). No physical characteristics of this disease in the acute form were found in the captured birds.

Other fungi that were isolated in abundance, mainly in fecal samples, were fungi of the order Mucorales, being the genus *Mucor* the most abundant. Fungi of this order have rapid growth and are commonly found in the soil and in decomposing plants. Mucor is the main genus of the order, they have simple or branched sporangiophores and form globular sporangia and lack rhizoids or have poorly developed rhizoids (De Souza *et al*., 2018; De Hoog *et al*., 2020; Cordeiro *et. al*., 2021). The sporangiospores of *Mucor* sp. vary in size and shape, with some irregular in shape (Freitas *et al*., 2021). The prevalence of Mucorales and *Cladosporium* spp. and *Fusarium* spp. found growing in feathers and droppings of wild birds are little known, therefore, there is a need to continue further studies.

When analyzing the samples of collected litter, we observed that the diversity of filamentous fungi was lower, only three genera were isolated (*Aspergillus*, *Penicillium* and *Fusarium*), when compared to the feces (sixteen genera) and feathers (nine genera) of surveyed birds (Table 1). Most microorganisms present in this environment are difficult to culture or depend on different microbiological techniques to obtain satisfactory growth. In this study, the technique of Vanbreuseghem (1952), was used to mainly isolate possible dermatophytes, fungi that can cause cutaneous mycoses in animals and humans (Vidal *et al*., 2017). According to Takahashi *et al*. (2011) and Vidal *et al*. (2017) the non-positivity of dermatophyte fungi suggests that the use of more sensitive methods for the identification of these fungi, such as those involving molecular biology, should be performed in order to enable an analysis of keratinophilic species present in the studied environments. Therefore, further necessary investigation to complement the classical techniques of fungal identification are molecular biology techniques for the identification of microorganisms.

Studies carried out by Labrador *et al*. (2021), quantifying the microbial abundance in the feathers of birds in the southern region of Spain, showed fungi as the main microorganisms. However, with the methodology used by the authors, it was not possible to identify the genera and morphospecies present in the samples. The most modern molecular biology techniques should complement the basic techniques of cultivation and identification, in order to identify specifically through the sequencing of nuclear genes, such as the ITS gene, which is the main gene used for molecular identification and phylogeny (Lima *et al*., 2017).

Birds may be involved in the transmission of fungal diseases in the following ways: as biological vectors and as mechanical vectors. Thus, in birds, fungi gain a diverse dispersion, since they are mechanically carried by their feathers or excreted in their feces (Hubalek, 2004). Fungi present in the soil, or on plant surfaces, are easily transferred to bird feathers. Inhalation of the spores or ingestion of grain containing the fungus are also routes of infection (Johansson *et al*., 2021). When inhaled or ingested, these fungi are excreted and dispersed in the environment, as reported by Akter *et al*. (2020), which analyzing the feces of migratory birds in Bangladesh, finding that these birds had an important role in the dissemination of filamentous fungi, especially *Aspergillus* spp. in

the environment, which could also be the case in our study.

Conclusion

A diversity of filamentous fungi of various genera was isolated from feathers and bird droppings, as well as from litter. The most reported genera were *Aspergillus*, *Mucor*, *Cladosporium*, *Fusarium*, *Penicillium,* and *Syncephalastrum*. According to the results found, it can be considered that the Atlantic Forest, even having suffered several impacts with the exponential growth and expansion of urban areas, is still a source of richness of fungal species. In addition, as this region is still poorly investigated regarding the fungal interaction with passerines, it is necessary that new mycological studies are carried out to survey the fungal species in the region, and molecular biology techniques are applied in order to find microorganisms such as keratinophilic fungi. The study of loads and fungi at different scales (between feathers, feces, and litter), allows us to suggest that the microorganisms that live in the feathers are the result of the arrival of fungi from the external environment to the bird. Other processes, such as microbial dispersion through feces, may play a role and should be further studied.

The continuity of the research is of great importance to elucidate whether the profile of the mycobiota is affected by anthropization and whether this profile interferes with the health of birds and the environment. This is the first study to report saprophytic filamentous fungi in wild birds and litter in the Itatiaia National Park in southeastern Brazil.

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