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Fungi isolated from wild birds in the Marambaia Island, Rio de Janeiro State, southeastern Brazil[1]

Jhon Lennon Genovez-Oliveira[2], Lucas A.S. Andrade[2], Mariana S. Oliveira[2], Viviane M. Lima[3], Bruno P. Berto[3]* and Águida A. de Oliveira[4]

ABSTRACT.- Genovez-Oliveira J.L., Andrade L.A.S., Oliveira M.S., Lima V.M., Berto B.P. & Oliveira Á.A. 2023. **Fungi isolated from wild birds in the Marambaia Island, Rio de Janeiro State, southeastern Brazil**. *Pesquisa Veterinária Brasileira 44:e07383, 2024*. Departamento de Biologia Animal, Instituto de Ciências Biológicas e da Saúde, Universidade Federal Rural do Rio de Janeiro, BR-465 Km 7, Seropédica, RJ 23897-000, Brazil. E-mail: bertobp@ufrrj.br

In Brazil, the Atlantic Forest has been suffering from deforestation, which has had impacts on its flora, fauna, and microbiota. However, the fungal diversity present in these environments is little known and studied. In this study, a total of 90 samples of 45 wild birds (45 feathers and 45 feces) were collected in Ilha da Marambaia, southeastern Brazil. Filamentous fungi isolated from these samples were identified through macroscopic and microscopic characteristics. Some isolates were identified by molecular biology using the PCR technique. *Acremonium, Alternaria, Aspergillus, Cunninghamella, Curvularia, Eurotium, Fusarium, Geotrichum, Neosartorya, Pestalotia, Paecilomyces, Penicillium*, *Rhizopus, Mucor* and *Syncephalastrum* were identified. These results indicate the presence of saprophytic fungi species in the feathers and feces of wild birds of the capture site. Further studies should be conducted to elucidate if the mycobiota profile modifies with anthropization and if it interferes with bird health and environmental recovery.

INDEX TERMS: Fungi, microbiota, passerines, Atlantic Forest.

RESUMO.- [Fungos isolados de aves silvestres na Ilha da Marambaia, Estado do Rio de Janeiro, Sudeste do Brasil.] No Brasil, a Mata Atlântica vem sofrendo com o desmatamento, que tem impactado sua flora, fauna e microbiota. No entanto, a diversidade fúngica presente nesses ambientes é pouco conhecida e estudada. Neste trabalho, um total de 90 amostras de 45 aves silvestres (45 penas e 45 fezes) foram coletadas na Ilha da Marambaia, Sudeste do Brasil. Fungos filamentosos isolados dessas amostras foram identificados por meio de características macroscópicas e microscópicas. Alguns isolados foram identificados por biologia molecular usando a técnica de PCR. Foram identificados *Acremonium, Alternaria, Aspergillus, Cunninghamella, Curvularia, Eurotium, Fusarium, Geotrichum, Neosartorya, Pestalotia, Paecilomyces, Penicillium, Rhizopus, Mucor* e *Syncephalastrum.* Esses resultados indicam a presença de espécies de fungos saprofíticos nas penas e fezes de aves silvestres do local de captura. Novos estudos devem ser realizados a fim de elucidar se o perfil da micobiota se modifica com a antropização e se interfere na saúde das aves e na recuperação ambiental.

TERMOS DE INDEXAÇÃO: Fungos, microbiota, pássaros, Mata Atlântica.

INTRODUCTION

In Brazil, a high variety of bird species can be found, being among the three countries with the greatest diversity of birds in the world. In this context, the Atlantic Forest, a tropical forest biome that covers the east, northeast, southeast, and south coast of Brazil, is among the top five in the list of world hotspots, even though its remaining area is less than 8% of its original extension (Schweizer et al. 2022). The loss and fragmentation of habitats and biopiracy are the main threats to its biodiversity, generating direct impacts on the fauna, flora, and microbiota (Lindström 1999). Within this biome, Marambaia Island is considered a biological reserve, which

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[2] Graduate Program in Animal Biology, Instituto de Ciências Biológicas e da Saúde (ICBS), Universidade Federal Rural do Rio de Janeiro (UFRRJ), BR-465 Km 7, Seropédica, RJ 23897-000, Brazil.

[3] Departamento de Biologia Animal, Instituto de Ciências Biológicas e da Saúde (ICBS), Universidade Federal Rural do Rio de Janeiro (UFRRJ), BR-465 Km 7, Seropédica, RJ 23897-000, Brazil. *Corresponding author: bertobp@ufrrj.br

[4] Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária (IV), Universidade Federal Rural do Rio de Janeiro (UFRRJ), BR-465 Km 7, Seropédica, RJ 23897-000, Brazil.

constitutes an environmental preservation area in accordance with Decree No. 9802 of March 12, 1987. The region is administered by the Brazilian Army, Air Force, and Navy, where they carry out armament experiments and military exercises. Moreover, around 430 remaining "quilombolas" are present in the area, making their living from fishing and agriculture. Access to the Island is restricted, which is only possible through navy vessels and with prior authorization (ICMBio 1987, Lima et al. 2020).

Pollination, insect control, and seed dispersal are examples of how birds act in the ecosystem chain (Howard 2003). These birds are among the animals that can act as reservoirs and dispersers for various microbial agents such as fungi, which can associate with feathers, when bumping into some substrate, and also with internal organs, entering through the air pathways and orally (Howard 2003, Reding 2003, Oliveira et al. 2022). Fungi are agents of the decomposition of organic matter and can be found in pollen, seeds, and soil (Simi et al. 2019, Kraisitudomsook et al. 2021). Furthermore, many of these are opportunistic, that is when in contact with immunocompromised hosts, they can cause disease (Pitt 1994, Simi et al. 2019).

The diversity of fungi present in the tropical environment is very high and most of them are still unknown (Oliveira et al. 2022). More studies about the frequency of environmental fungi become important since most of them have an opportunistic profile (Oliveira et al. 2022). Therefore, it is of great importance to define the profile of the mycobiota residing in these places, as well as to establish the incidence of filamentous and/or opportunistic fungi.

When it comes to filamentous fungi, the most common descriptions are in broilers (Sugiharto 2019, Hamza & Gunyar 2022) in domestic birds, such as pigeons (Madsen et al. 2023), and captive birds (Talbot et al. 2018). However, in free-living birds, this interaction remains poorly studied.

In this scenario, this study aimed to establish the incidence of filamentous fungi from feathers and feces of birds in localities of Marambaia Island, in the Southeast of Brazil, to understand the diversity and frequency of fungal species in this habitat.

MATERIALS AND METHODS

Animal Ethics. Field-collecting permits were issued by the "Instituto Chico Mendes de Conservação da Biodiversidade" (Chico Mendes Institute for Biodiversity Conservation - ICMBio), through the "Sistema de Autorização e Informação em Biodiversidade" (Biodiversity Authorization and Information System - SISBIO) under license number 70132, and Animal Ethics Committee (CEUA) of the "Universidade do Grande Rio" (UNIGRANRIO) under protocol number 021/2019.

Study site and sample collection. This study was conducted on Marambaia Island, a protected area located in the State of Rio de Janeiro, in the Southeast of Brazil (22°26'17" S; 44°37'33" W). The expeditions were carried out in May, June, and July 2021. The captures took place three days per month and 10 mist nets were used, totaling 180 meters, and they remained open from 5 a.m. to 5 p.m., that is, 12 hours per day and 36 hours per month. A total of 45 birds of different species were captured (Table 1). The 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 into the same environment where they were captured. All samples were properly labeled, packed in thermal bags at room temperature, and transported to the Laboratory of Mycology and Mycotoxicology at the "Universidade Federal Rural do Rio de Janeiro" (UFRRJ).

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 seven 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 seven days (Nardoni & Mancianti 2021). For the identification of the fungi grown on the plates and manes, the following was 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 (Samson et al. 2000, Sidrim & Rocha 2004, De Hoog et al. 2020).

Molecular identification. DNA was extracted from a total of eight strains of different species, identified according to the morphological taxonomy, and the results of these identifications were compared. After isolating and obtaining pure cultures, total DNA extraction was performed using the commercial kit DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's recommendations. Then, amplification was performed using the polymerase chain reaction (PCR) technique, in the region corresponding to the internal transcribed spacer (ITS) ITS1 – 5,8S – ITS2. The PCR reaction contained 12.5µl of GoTaq® G2 Hot Start Colorless Master Mix (Promega Labs, São Paulo, Brazil) (1×), 0.25µl of each primer (0.2µM), 9µl of nuclease-free water and 3µl of DNA (for the primary reaction) or 3µl primary PCR product (for the secondary reaction). This reagent mixture was subjected to amplification with a temperature profile that consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes. At the end of the 35 cycles, a final extension was performed at 72°C for 10 minutes (Lima et al. 2017). The primers used for amplification were ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al. 1990). The PCR amplicons were purified using the Qiagen MinElute PCR Purification (Qiagen, São Paulo, Brazil). All PCR amplicons were sequenced using the PCR forward and reverse primers by Ludwig Biotechnology, where an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems, Foster City, California) was used for Sanger sequencing.

RESULTS

A total of 90 samples from 45 wild birds captured were collected, which included feathers (n=45) and feces (n=45) (Table 1). From each sample, it was possible to isolate one or more filamentous colonies, while in others there was no growth and, therefore, the number of isolated fungi does not correspond to the total number of samples. Out of a total of 68 isolated fungi, 15 genera were identified, as shown in Table 2. The genera with a greater number of occurrences were *Mucor* and *Fusarium*, followed by *Syncephalastrum*, *Penicillium*, and *Aspergillus.*

The vast majority of fungal species isolated from the feathers and feces of birds in this study are considered to belong to the saprophytic fungi genera, occasionally being opportunistic pathogens. Based on the phenotypic characteristics and corresponding taxonomic keys, we can verify that, among the isolated samples, 15 genera were identified, namely *Acremonium, Alternaria, Aspergillus, Cunninghamella, Curvularia, Eurotium, Fusarium, Geotrichum, Neosartorya*, *Pestalotia*, *Paecilomyces*, *Penicillium*, *Rhizopus, Mucor* and *Syncephalastrum* (Table 2).

Table 1. Bird species, samples, and isolated fungi

Family	Species	Feces	Feathers
Tyrannidae	*Myiarchus ferox*	*Fusarium* sp.	-
	M. ferox	*Fusarium* sp.	-
	Myiozetetes similis	*Fusarium* sp.	-
	M. ferox	*Eurotium* sp., *Fusarium* sp.	-
	M. ferox	*Syncephalastrum* sp.	-
	M. ferox	*Syncephalastrum* sp. *Fusarium* sp.	-
	M. ferox	*Syncephalastrum* sp.	*Penicillium* sp.
	M. ferox	*Syncephalastrum* sp.	*Syncephalastrum* sp.
	M. ferox	-	-
	M. similis	*Mucor* sp.	-
	M. similis	*Mucor* sp.	-
	Elaenia flavogaster	*Mucor* sp.	-
	M. ferox	*Penicillium* sp., *Pestalotia* sp.	-
	Tyrannus melancholicus	*Fusarium* sp.	*Syncephalastrum* sp.
Turdidae	*Turdus rufiventris*		*Cunninghamella* sp.
	T. rufiventris	*Pestalotia* sp., *Fusarium* sp.	
	T. rufiventris	*Mucor* sp.	*Fusarium* sp., *Geotrichum* sp.
	Turdus amaurochalinus	*Mucor* sp.	-
	Turdus leucomelas	*Mucor* sp.	-
	T. rufiventris	*Syncephalastrum* sp.	-
	T. leucomelas	*Mucor* sp., *Syncephalastrum* sp.	-
	T. leucomelas	*Syncephalastrum* sp.	*Mucor* sp.
Columbidae	*Leptotila verreauxi*	-	*Mucor* sp., *Fusarium* sp., *Mucor* sp., *Rizopus* sp.
	L. verreauxi	*Acremonium* sp.	
	Columbina talpacoti	*Rhizopus* sp.	*Penicillium* sp.
	L. verreauxi	-	-
	Leptotila rufaxilla	*Fusarium* sp., *Paecilomyces* sp.	-
	L. verreauxi	*Fusarium* sp.	-
Hirundinidae	*Progne chalybea*	*Alternaria* sp.	*Fusarium* sp.
	P. chalybea	*Rhizopus* sp.	-
	P. chalybea	*Mucor* sp.	*Aspergillus* sp.
	P. chalybea	-	*Fusarium* sp., *Aspergillus* sp.
	P. chalybea	-	*Rhizopus* sp.
Vireonidae	*Vireo chivi*	*Eurotium* sp., *Fusarium* sp.	-
	V. chivi	*Mucor* sp.	-
	V. chivi	*Curvularia* sp., *Mucor* sp.	*Penicillium* sp., *Aspergillus* sp.
	V. chivi	*Mucor* sp.	-
	V. chivi	*Mucor* sp.	-
Thraupidae	*Ramphocelus bresilia*	*Penicilium* sp.	-
	R. bresilia	*Mucor* sp.	-
	R. bresilia	*Acremonium* sp.	-
	Thraupis palmarum	*Acremonium* sp., *Fusarium* sp.	*Curvularia* sp.
Alcedinidae	*Chloroceryle americana*	*Syncephalastrum* sp.	-
Icteridae	*Molothrus bonariensis*	*Fusarium* sp.	-
Picidae	*Veniliornis maculifrons*	*Neosartorya* sp.	-
TOTAL	45	48	20

DISCUSSION

It should be noted that studies involving the mycobiota present in wild birds, mainly Passeriformes, despite their great importance, are scarce, and, in Marambaia Island, no survey of the mycobiota present in the feathers or feces of these animals has been conducted so far. This seems to be the first study to report saprophytic fungi in wild birds on Marambaia Island.

The Tyrannidae family was the most prevalent in the capture (Fig.1). It is important to point out that tyrants are one of the most diverse and numerous groups of birds worldwide and, consequently, also in the neotropical region (Chaves et al. 2008). These birds have spread to every conceivable habitat (Ridgely & Tudor 1994) and they adapt to a wide variety of ecological niches as they occupy all different vertical strata within tropical forests (Sick 1997). When separately analyzing fungal isolates from this family, we noticed that the most prevalent genus was *Fusarium*, found only from the feces of these animals and none from the feathers, which suggests the presence of these fungi in the diet and gastrointestinal tract of these birds. The vast majority of tyranids are insectivores and a few feed on fruits (Brum et al. 2012). These results reinforce the need for further studies involving the presence of fungi and the correlation with the food consumed by these animals.

The genus *Fusarium* sp. is common in grains and commercial feed used for captive birds, as reported by Köptcke et al. (2021), who evaluated contamination by fungi and their mycotoxins in feed offered to birds of the species *Nymphicus hollandicus*, popularly known as cockatiels. Over six months, the feed intended for consumption by these birds was collected for a mycological analysis, resulting in a high fungal activity of the genus *Fusarium*.

Birds can carry pollen, seeds, small parasites, and even fungi on their feet, feathers and beak, thus acting as dispersers in an ecologically balanced environment (Hubalek 2004). Therefore, it is possible to assume the importance of isolating fungi from the feces and feathers, as it was performed in the present study.

Among the 15 genera reported, the most frequent ones in this research were *Mucor*, *Fusarium*, and *Syncephalastrum*, followed by *Penicillium*, *Aspergillus*, and *Rhizopus* (Fig.2-7), and when analyzing the total number of fungi present in the substrates (feces and feathers), we observed a greater number of isolated genera in the feces than in the feathers (Table 1). Similar results were found by Oliveira et al. (2022) who, when studying the fungal diversity present in the birds of the Itatiaia National Park in Brazil, reported the presence of *Aspergillus* spp., *Mucor* spp. *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp. and *Syncephalastrum* spp. The same authors showed a higher prevalence of fungi in the feces than in the feathers of the captured birds.

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

Fungi	Feces	Feathers
Acremonium	3	-
Alternaria	1	-
Aspergillus	-	3
Cunninghamella	-	1
Curvularia	1	1
Eurotium	2	-
Fusarium	12	4
Geotrichum	-	1
Mucor	13	3
Neosartorya	1	
Penicillium	2	3
Pestalotia	2	-
Paecilomyces	1	-
Rhizopus	2	2
Syncephalastrum	8	2
TOTAL	48	20

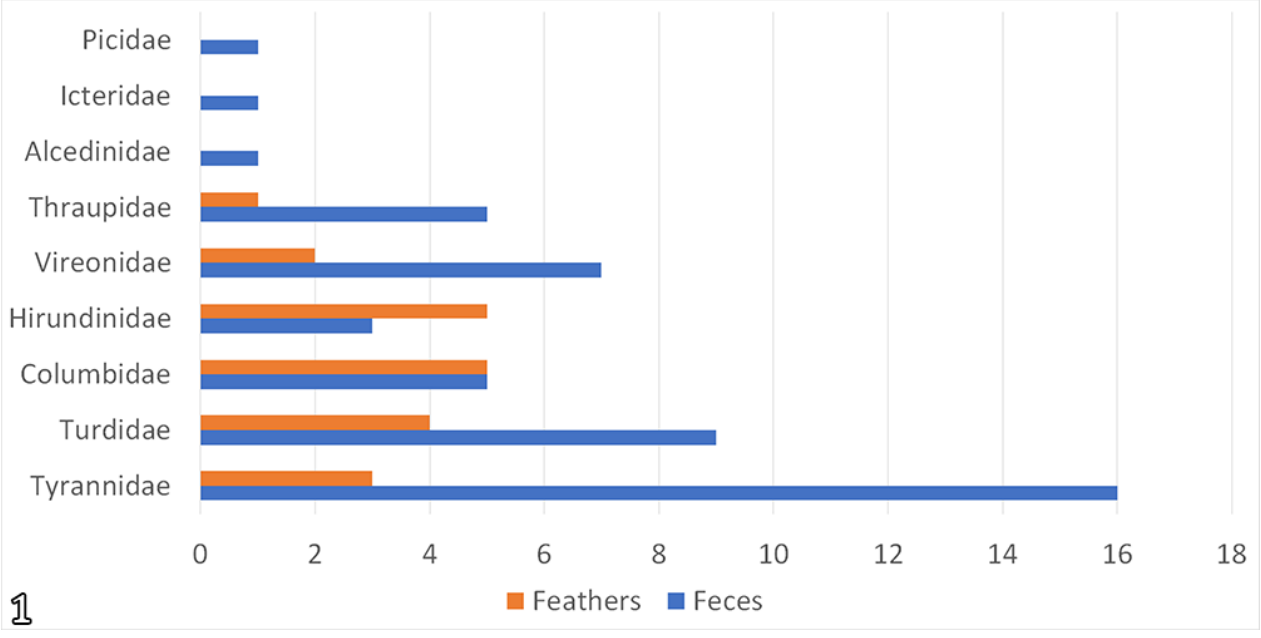

Fig.1. Distribution of fungi isolated from the collected substrates, according to the bird family.

Another genus largely found was *Mucor*, belonging to the order of Mucorales. These fungi have fast-growing and woolly colonies. *Mucor* is the main genus of the order, which has simple or branched sporangiophores, forms globular sporangia, and does not have rhizoids (De Hoog et al. 2020, Freitas et al. 2021). Further, when analyzing the presence of this fungus in the substrates, we noticed that it was reported in greater quantity in the feces than in the feathers, as also evidenced by Oliveira et al. (2022), reinforcing the need for further studies involving the feeding of these animals.

According to Oliveira et al. (2022), it is difficult to observe the dispersion of filamentous fungi through feces in the wild environment, since when the feces are dispersed, whether, in soil or litter, they generate cross-contamination with fungi that were present in these substrates, making it difficult to know its origin. Thus, the capture methodology of this study allows a more precise identification, since the feces are collected and kept in sterilized paper envelopes, therefore, avoiding cross-contamination. These results ensure that these fungi were present in the feces of the birds and that they are by them dispersed into the environment. Other studies have already reported this dispersion using different methodologies, such as Correia et al. (2019). The authors analyzed the feces with intact seeds, which were placed in sterilized soil and kept for four months in a protected environment to avoid contamination. As a result, seven seedlings of *Rubus ulmifolius* obtained from four independent feces of *Erithacus rubecula* and *Sylvia melanocephala*, were colonized by arbuscular mycorrhizal fungi.

As for the genetic analysis, nucleotide sequences were obtained from the regions of interest (ITS) ITS1 – 5.8S – ITS2, and all these sequences were subjected to species-level identification through comparison with sequences deposited in the GenBank database, which made it possible to identify species such as *Syncephalastrum racemosum*, *Pestalotiopsis microspora*, *Alternaria alternata*, *Penicillium herquei*, *Trichoderma asperellum* and *Fusarium* sp. (Table 3). Despite the sequenced regions being widely used in several studies for the characterization of fungal species, for some isolates, these regions were not sensitive enough for amplification. Because it covers the diversity of the mycobiota of the Atlantic Forest, we may be dealing with new species that have not yet been described or a primer that does not have enough similarity for amplification.

The most modern techniques of molecular biology should complement (and not replace) the basic techniques of cultivation and identification to avoid identification errors, using morphological taxonomic keys and sequencing of nuclear genes, such as the ITS gene, which is the main gene used for molecular identification and phylogeny (Lima et al. 2017). Studies conducted by Labrador et al. (2021) verified the prevalence of Kingdom Fungi in relation to other microorganisms in the feathers of birds from southern Spain, showing fungi as the main microorganisms. However, the methodology used by the authors did not prioritize the description of morphospecies.

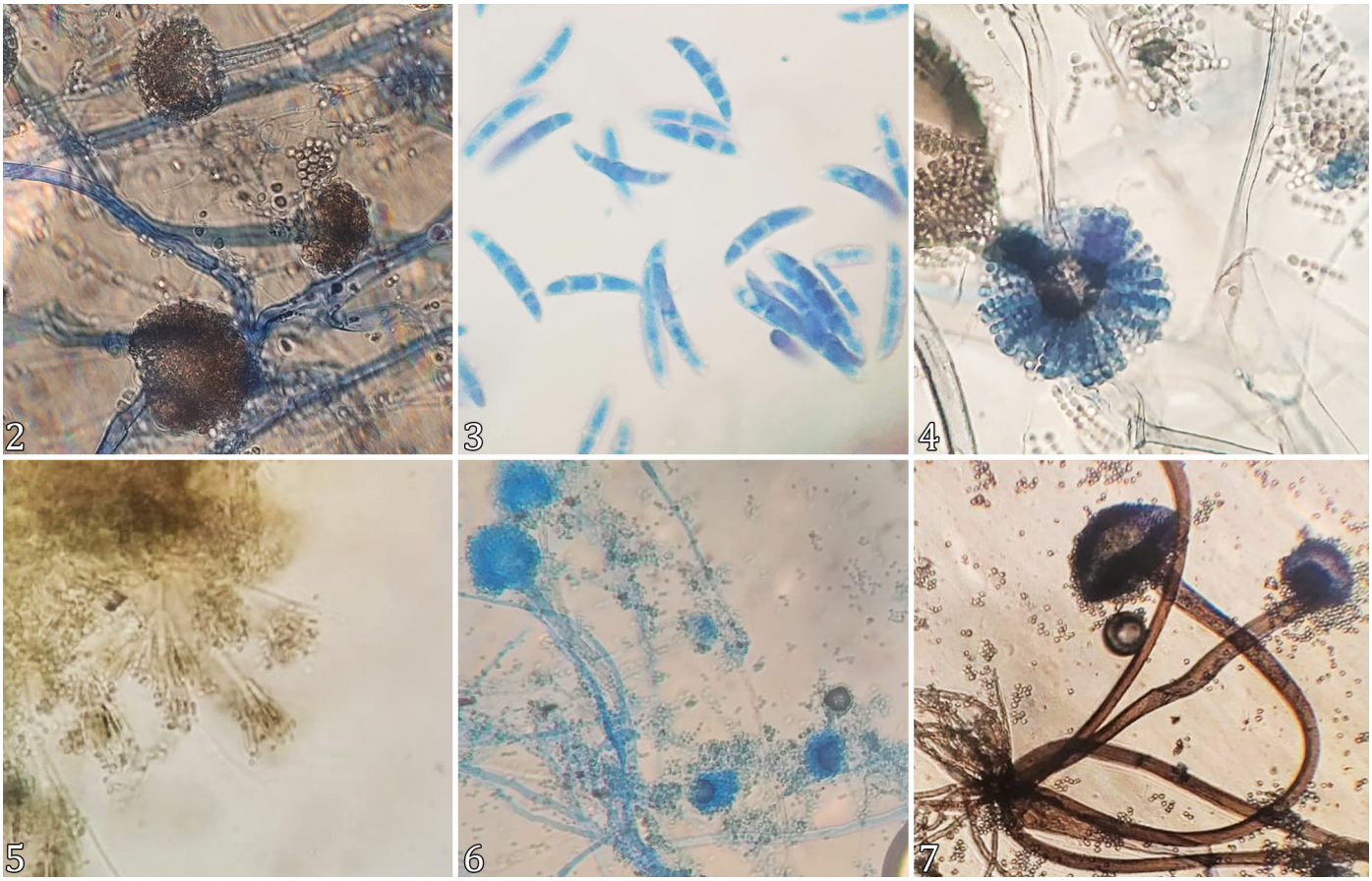

Fig.2-7. Light microscopy of isolated fungi. (**2**) *Mucor* sp. with lactophenol cotton blue. (**3**) *Fusarium* sp. with lactophenol cotton blue. (**4**) *Syncephalastrum* sp. with lactophenol cotton blue. (**5**) *Penicillium* sp. with sodium hydroxide (20%). (**6**) *Aspergillus* sp. with lactophenol cotton blue. (**7**) *Rhizopus* sp. with lactophenol cotton blue. Isolate from feces.

Table 3. Species identified by traditional taxonomy and molecular biology

Primer	Identification blast	Identity	Morphological analysis
ITS1	*Alternaria* sp.	99.81%	*Alternaria* sp.
ITS4	*Alternaria alternata*	100%	
ITS1	*Fungal endophyte culture*	100%	*Pestalotiopsis* sp.
ITS4	*Pestalotiopsis microspora*	99.61%	
ITS1	*Syncephalastrum racemosum*	100%	*Syncephalastrum* sp.
ITS4	*S. racemosum*	99.31%	
ITS1	*Fungal* sp.	100%	*Syncephalastrum* sp.
ITS4	*S. racemosum*	98.97%	
ITS1	*Trichoderma asperellum*	99.65%	*Mucor* sp.
ITS4	There was no similarity		
ITS1	*Penicillium herquei*	98.84%	*Penicillium* sp.
ITS4	*P. herquei*	99.82%	
ITS1	*S. racemosum*	99.82%	*Aspergillus* sp.
ITS4	There was no similarity		
ITS1	*Fusarium* sp.	96.96%	*Fusarium* sp.
ITS4	*Fusarium* sp.	100%	

CONCLUSIONS

A diversity of filamentous fungi of various genera was isolated from the feathers and feces of birds. The most reported genera were *Mucor*, *Fusarium*, *Syncephalastrum*, *Penicillium*, *Aspergillus*, and *Rhizopus*.

According to the results found, it can be considered that the Atlantic Forest, even though it has suffered several impacts with the exponential growth and expansion of urban areas, is still a rich source of fungal species. The creation of new primers should be encouraged to shed light on a greater range of fungal species.

Moreover, as this region is still poorly investigated regarding the interaction of fungi with passerines, it is necessary that new mycological studies be conducted to survey the fungal species in the region, and molecular biology techniques be concomitantly applied (polyphasic taxonomy) to assist in the identification of these microorganisms.

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Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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