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Research Article

MOLECULAR IDENTIFICATION OF LEAF SPOT-LIKE PATHOGENS INFECTING APPLES OF SOE CULTIVAR IN TIMOR ISLAND

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ABSTRACT

Apples of the Soe cultivar are a major horticultural crop on Timor Island, East Nusa Tenggara Province, Indonesia. However, their cultivation and productivity are significantly hindered by leaf spot disease. This disease causes severe damage to trees, impairing their ability to photosynthesize and consequently reducing fruit production. Various training initiatives have been implemented to improve fungal pest control in apple cultivation, yet expected results have not been achieved, largely because the specific pathogen had not been identified. Therefore, in the current study, leaf samples showing leaf spot symptoms were collected from apple orchards in five villages of Timor Island: Pubasu, Tunua, Tubuhue, Tobu, and Binaus. The samples underwent DNA extraction, followed by amplification through PCR. The amplified DNA was sequenced and analyzed using BLAST to identify the exact pathogen species. Molecular analysis revealed three primary pathogens responsible for leaf spot in the Soe apple cultivar: *Aspergillus flavus*, *A. fumigatus*, and *Fusarium solani*. The study provided molecular identification and characterization of the major pathogens causing leaf spot in Soe apples on Timor Island. Identifying these pathogens lays the groundwork for developing more targeted and effective disease management strategies, empowering farmers to better manage and potentially eradicate this disease.

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INTRODUCTION

The Soe cultivar apple is a unique and valuable agricultural commodity on Timor Island, East Nusa Tenggara Province, Indonesia. Once nearly extinct (Fuah et al., 2013), efforts to revive its cultivation began in 2016. While promising progress has been made, the cultivation process remains suboptimal, especially in scaling production to meet consumer demand. One of the primary obstacles to enhancing Soe apple productivity is the ongoing challenge of managing infections caused by pathogenic microorganisms, particularly fungi. Despite various pest control strategies, including farmer training programs on fungal management, results have been disappointing.

The tropical climate, with its high humidity, exacerbates these challenges by creating ideal conditions for fungal

pathogens. Many of these pathogens have short latent periods, leading to rapid spread and secondary infections (Liang et al., 2022). Local farmers have faced substantial setbacks, as traditional pesticide applications, often formulated without precise knowledge of pathogen behavior, have proven ineffective. These difficulties highlight the need for a more targeted and scientifically informed approach to pest and disease control. Although farmer training programs have aimed to address these issues by educating growers on fungal management techniques, limitations persist. Farmers frequently lack access to advanced tools and methods for accurate pathogen identification and control. Moreover, environmental factors such as rainfall patterns and soil conditions add further complexity to effective disease management.

A promising solution lies in implementing site-specific pest and disease management strategies. However, effective control systems require accurate identification of the specific pathogenic microorganisms involved (Srivastava et al., 2020). Molecular-based identification methods, particularly DNA sequencing, provide the precision needed to identify the exact species responsible for infections, thereby significantly enhancing the effectiveness of disease control measures (Nizamani et al., 2023).

Fungi are recognized as major pathogens in horticultural crops, causing diseases such as leaf spot in apples (Megavitry et al., 2022). Leaf spot symptoms include blackish-brown acervuli on leaves, which release fungal conidia, spreading the infection (Back et al., 2015). This infection can result in chlorosis, defoliation, and a decline in the photosynthetic capacity of plant, potentially reducing fruit yield by up to 100% if left unmanaged (Sastrahidayat and Nirwanto, 2016). Several fungal species, including *Marssonina coronaria*, *Alternaria mali*, *Botryosphaeria obtusa*, and *Glomerella cingulata*, are known to cause apple blotch-like symptoms (Back et al., 2015; Toome-Heller et al., 2018).

Field observations on Timor Island suggest that multiple fungal species are contributing to the decline in Soe apple productivity. Preliminary assessments of soil and surrounding plant conditions support this hypothesis (Baranwal et al., 2019). Leaf samples collected from five villages (Pubasu, Tunua, Tubuhue, Tobu, and Binaus) were analyzed through DNA extraction, PCR amplification, and sequencing to identify the specific fungal species responsible. This study represents the first effort to apply molecular techniques for pathogen identification in Soe apple cultivation, aiming to establish a foundation for more effective and targeted disease management strategies.

MATERIALS AND METHODS

Materials

The materials used in this study included infected apple leaves showing fungal symptoms and various laboratory reagents. These reagents included sodium hypochlorite for sterilization, distilled water, Potato Dextrose Broth (PDB) and Potato Dextrose Agar (PDA) media for fungal cultivation, TE buffer solution used during DNA extraction, water agar, extraction buffer, sodium acetate, isopropanol, and 70% ethanol for DNA precipitation and purification. Each reagent and material was essential for either isolating and cultivating the fungi or processing their DNA for identification.

Procedures

Apple leaf sampling

Apple leaf samples were collected aseptically from five apple orchards in the South Central Timor district (Tubuhue, Binaus, Tunua, Tobu, and Pubasu). Aseptic sampling was conducted to prevent external contamination, ensuring that only fungi present on the apple leaves were analyzed. The sampled leaves exhibited blotch-like symptoms, characterized by blackish-brown acervuli spots, indicating potential fungal infection (Back et al., 2015).

Sterilization of apple leaf samples

To minimize contamination from non-pathogenic surface microbes, the surfaces of apple leaf samples were sterilized using 9% sodium hypochlorite solution for 2 min, followed by three rinses with sterile distilled water. Sodium hypochlorite is a widely used disinfectant in microbiology, effectively eliminating surface pathogens while preserving the integrity of internal fungal structures. The repeated rinsing with sterile water ensures the removal of any residual bleach, which could otherwise inhibit fungal growth during subsequent culturing (Lee et al., 2011).

Fungal isolation and purification

Small sections (3-5 mm) of the sterilized leaf acervuli were placed onto water agar and incubated at 25°C in the dark for two weeks. The dark environment inhibits the growth of photosynthetic organisms and aids in isolating fungal colonies. Water agar, a basic medium, supports fungal growth while minimizing the

proliferation of bacterial contaminants. Once fungal colonies appeared, they were purified by subculturing onto PDA, a nutrient-rich medium designed to promote fungal growth and facilitate the isolation of distinct fungal species (Sastrahidayat and Nirwanto, 2016).

DNA extraction

Fungal DNA extraction was performed using a modified version of the method described by Abd-Elsalam et al. (2003). Fungi were cultured in 50 ml of PDB for 72 h on a shaker to promote optimal growth and biomass production. After centrifugation at 10,000 rpm, fungal mycelia were collected, washed with TE buffer to stabilize the DNA and remove contaminants, and then ground to release cellular contents.

The extraction buffer contained Tris-HCl to maintain a stable pH, NaCl to stabilize DNA and disrupt protein interactions, EDTA to chelate divalent cations and inhibit DNases, and SDS, a detergent that lyses cells and dissolves membranes. Sodium acetate was added to precipitate proteins and other contaminants, and the supernatant containing the DNA was transferred to a new tube for further processing.

DNA precipitation was achieved using isopropanol, which causes the DNA to aggregate and form a visible pellet upon centrifugation. A 70% ethanol wash was used to remove salts and other impurities that could interfere with subsequent PCR amplification. Finally, the DNA pellet was air-dried and resuspended in TE buffer for storage at -20°C, ensuring the integrity of DNA and protection from degradation.

Amplification

The internal transcribed spacer (ITS) region of fungal rDNA was amplified using PCR. The ITS region is highly variable among fungal species and is widely used as a genetic marker for species identification. Primer pairs ITS1 (forward) (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (reverse) (5'- TCCTCCGCTTATTGATATGC-3') were selected because they target conserved regions flanking the ITS region, enabling amplification across a broad range of fungal species (White, 1990).

The PCR reaction began with an initial denaturation step at 94°C to unwind the DNA strands, followed by 35 cycles of denaturation, annealing (binding of the primers to the target DNA at 55°C), and elongation (synthesis of the new DNA strand by Taq polymerase at 72°C). This cycling process ensures exponential amplification of the target DNA. A final elongation step at 72°C ensures that any incomplete DNA fragments are fully extended (White, 1990).

DNA sequencing

The amplified DNA was sent to First Base (Malaysia) for sequencing. Sequencing is essential to determine the exact nucleotide sequence of the amplified ITS region, which can then be compared against known fungal sequences in public databases (Vertygo et al., 2023).

Data analysis

The resulting sequences were analyzed using the BLAST (Basic Local Alignment Search Tool) program from the NCBI database to identify the fungal species. BLAST compares the query sequence to sequences in the database to find the closest match, enabling precise identification of the fungal pathogens responsible for apple leaf infections (Altschul et al., 1990).

RESULTS

Isolation of fungal pathogens

During the isolation process, five isolates were obtained from each village, primarily responsible for causing leaf spot-like symptoms. WA was initially used to select the fungal pathogens responsible for these diseases. To infect the leaves, the pathogens must first break down cellulose, which is predominantly found in plant tissue (Shirsath et al., 2018). Water Agar contains only water and cellulose fiber from the agar, providing an ideal carbon source for cellulose-degrading fungi, including those capable of causing leaf spot-like symptoms (Cheng et al., 2021). Purification was carried out using PDA media, where the fungal colonies were inoculated until purified isolates were obtained.

Identification of fungal pathogens

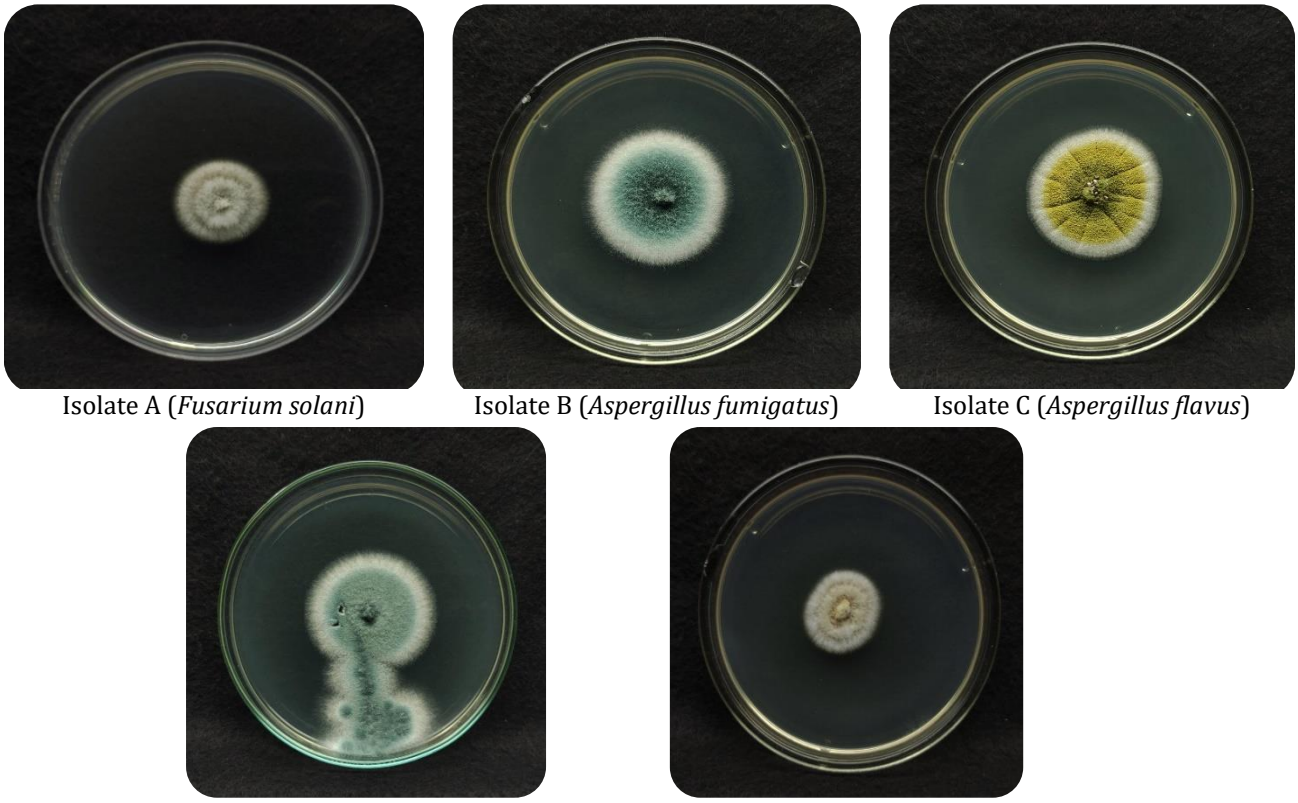
The purified isolates were then subjected to DNA extraction and sequencing. DNA sequences were identified through a BLAST search on the NCBI database. Based on the molecular identification, the five main isolates were classified into three species, as shown in Table 1 with their macroscopic appearance presented in Figure 1.

DISCUSSION

The identification of *Aspergillus flavus*, *A. fumigatus*, and *Fusarium solani* as the major pathogens responsible for leaf spots in *Soe* apple cultivars offers valuable insights into the nature of the disease on Timor Island. These findings enhance our understanding of the pathogen diversity associated with leaf spot disease and can aid in developing effective pest and disease management strategies.

Table 1. Molecular identification of fungal pathogens isolated from apple leaves of Soe cultivar.

Isolate Code	GenBank Isolate	Location in District	Accession number	E-value	Homology (%)
A	*Fusarium solani*	Pubasu	MH370554.1	0,0	99,63
B	*Aspergillus fumigatus*	Tunua	MF379664.1	0,0	99,47
C	*Aspergillus flavus*	Tubuhue	LN482516.1	0,0	99,65
D	*Aspergillus fumigatus*	Tobu	MN634497.1	0,0	100,00
E	*Fusarium solani*	Binaus	MH370553.1	0,0	100,00



Isolate A (*Fusarium solani*) Isolate B (*Aspergillus fumigatus*) Isolate C (*Aspergillus flavus*)

Isolate D (*Aspergillus fumigatus*) Isolate E (*Fusarium solani*)

Figure 1. Macroscopic morphology of pathogenic fungi isolated from leaves of the Soe apple cultivar.

F. solani is known to be one of the most significant plant pathogens, affecting many important agricultural crops, including apple trees (*Malus domestica*) (Coleman, 2016). While primarily a soil-borne fungus, *F. solani* can infect various parts of the apple tree, including the roots, trunk, branches, and leaves (Wang et al., 2010). In apple trees, this fungal infection is commonly associated with leaf spot disease (Xiang et al., 2021). Infection by *F. solani* typically manifests as small, dark-colored lesions or spots on the leaves. Initially, these spots may appear water-soaked or necrotic, and as the disease progresses, they can enlarge and merge, leading to defoliation and reduced photosynthetic capacity (Cui et al., 2022). Severe infections can disrupt normal cell division processes. Infected cells may experience DNA damage, leading to cell cycle arrest while repair mechanisms attempt to correct the damage. If the damage is extensive, it can inhibit cell division, affecting tissue growth and development, which in turn impacts overall plant health and productivity (Agustina et al., 2021). In severe cases, infections can weaken the tree, hinder fruit development, and potentially cause yield losses (Zhong and Zhao, 2020).

It is important to note that *F. solani* infection is often favored by specific environmental conditions, such as high humidity, warm temperatures, and wounds or injuries to the tree. The fungus can enter the apple tree through natural openings or through wounds caused by

pruning, insect feeding, or other mechanical damage (Elvira-Recuenco et al., 2020). *F. solani* is also known for its ability to survive during dormancy and the initial vegetative growth phase of apples by residing both externally and internally in the twigs of the tree (Gelain et al., 2022).

The biology of *F. solani* includes several virulence factors, such as the production of mycotoxins like trichothecenes and fusaric acid. These toxins inhibit protein synthesis in plant cells, leading to necrosis and cell death (Shi et al., 2016). Additionally, the fungus secretes cell wall-degrading enzymes, including pectinases, cellulases, and proteases, which break down plant cell walls and facilitate tissue invasion (Mezzomo et al., 2019). Other virulence factors, such as the production of chitinases and melanin, help the fungus evade plant defense mechanisms and enhance its survival in hostile environments (Laien and Mohammadi, 2020; Chaves-González et al., 2024).

Ecologically, the pathogen thrives in warm temperatures between 25°C and 30°C and prefers moist soils. However, its resilient spore structures enable it to persist under a range of environmental conditions. This adaptability makes it a persistent threat to crops in many agricultural settings (Saremi et al., 1999).

Management strategies for *F. solani* infection include cultural practices such as maintaining proper tree hygiene, implementing good irrigation practices, and avoiding excessive soil moisture around the base of the tree (Jiang and He, 2021). Fungicide application can also be considered, following sensitivity testing, as different strains show varying responses to the fungicide-inhibitory effects (Coronel et al., 2022). Additionally, a study conducted in an apple orchard in South Tyrol, Italy, linked the initial incidence and severity of leaf blotch with low concentrations of magnesium, sulfur, and especially manganese (Prechsl et al., 2023). Therefore, promoting overall tree health through balanced nutrition and regular monitoring can help mitigate the impact of *F. solani* and other diseases (Bahadur, 2021). Liu et al. (2023) found that, at the molecular level, *Fusarium*-infected apples exhibit high expression of the gene MdERF114, which is involved in lignin deposition to mediate resistance to such pathogenic infections. This finding could serve as a foundation for developing genetic recombination strategies, such as *Agrobacterium*-mediated transformation (Lv et al., 2019), for more effective and specific *Fusarium* control programs.

Genetically, members of this species are also known to encode host-specific virulence factors that may assist in their invasion of a wide range of plant hosts (Gherbawy et al., 2021; Sabahi et al., 2023). To successfully reside inside the host, this species has developed several strategies to circumvent the defense mechanisms of the host, particularly in relation to the antifungal compounds synthesized by the host. These strategies include the ability to break down or modify the compounds to a less virulent state, as well as to exclude the compounds from entering the cell and causing further physiological damage (Coleman, 2016). Yan et al. (2018) observed that apple seedlings infected with *F. solani* exhibited morphological effects, such as leaf wilting. Further physiological analysis revealed a correlation with the inhibition of photosystem activities (especially Photosystem I), an increase in proline amino acid content, and a decrease in leaf water content. Photosystems are a group of pigments essential for photosynthesis, which supports plant growth and development (Vertygo, 2021). The higher proline accumulation indicated a decrease in water content, as proline serves as an osmolyte that mitigates the effects of water deficit (Chun et al., 2018). In North-Western China, members of the *Fusarium* genus, including *F. solani*, were found to be significant factors in the decline of wild apple trees on the northern slope of the Tian Shan Mountains. Similar results were reported in apple nurseries in Tunisia, where *F. solani* infected apple seedlings, causing stem discoloration and leaf abscission in over 75% of the plants (Mannai et al., 2018). In the Pubasu and Binaus districts, where this pathogen was most dominant, infected trees showed symptoms of brownish spots on the leaves, which spread throughout the organ, affecting other leaves and twigs, eventually leading to their abscission. However, one limitation of this finding is the potential for misidentification of closely related *Fusarium* species, as many species within the genus exhibit similar pathogenic traits. Although ITS sequencing is well-established for species identification, future studies could benefit from using multilocus sequencing (MLS) or whole-genome sequencing to ensure accurate identification at the strain level (Uelze et al., 2020).

Another pathogen identified in apple orchards on Timor Island was *A. fumigatus*. This pathogenic fungus was predominantly found in the districts of Tunua and Tobu. *A. fumigatus* is primarily known as an opportunistic

human pathogen and is not typically associated with causing diseases in apple trees (Pennerman et al., 2020). In general, *Aspergillus* species are more commonly linked to post-harvest diseases of fruits and vegetables rather than direct infections of trees (Taniwaki et al., 2018). Although *Aspergillus* species can be found in various environments, including agricultural settings, their ability to infect and cause significant damage to apple trees is rare. However, these species are known to possess genes that express enzymes capable of degrading plant materials, such as lignin, pectin, hemicelluloses, and celluloses (Liu et al., 2013). Moreover, *Aspergillus* is commonly found as a terrestrial fungus inhabiting the topsoil of many agricultural sites (Barber et al., 2020). Therefore, an opportunistic infection in apple trees is still a plausible explanation.

Another species of the *Aspergillus* genus, *A. flavus*, was found to infect many apple trees in the Tubuhue district. In agricultural settings, *A. flavus* is particularly concerning due to its ability to produce toxic secondary metabolites known as aflatoxins. These toxins can contaminate food and feed crops, posing health risks to both humans and animals, even when consumed in relatively small amounts (Dhakal et al., 2023). Although *A. flavus* is widely distributed in the environment and can colonize a variety of substrates, including fruits, it is not typically associated with direct infections of apple trees and is not considered a common pathogen in apple orchards. Instead, it is mainly linked to the contamination of agricultural commodities such as grains, nuts, and oilseeds (Smiri et al., 2021). A study by Afandhi et al. (2017) successfully isolated endophytic fungi from young, mature, and old apple leaves, finding that *Aspergillus* was the most abundant genus. However, it was suggested that, as an endophytic fungus, *Aspergillus* had a beneficial relationship with the host plant, helping it combat other pathogenic microorganisms. This contrasts with the observations of the infected apples in Timor Island, where *Aspergillus* species were identified as the pathogenic fungi. *Aspergillus* species are known to act as opportunistic pathogens, invading susceptible host plants under unfavorable environmental conditions such as drought and extreme temperatures (Dolezal et al., 2014; Poudel et al., 2021). This could explain the infection of apple trees on Timor Island by *Aspergillus* species.

The identification of *A. fumigatus* and *A. flavus* as pathogens in apple trees is somewhat unexpected, as these species are more commonly associated with post-harvest diseases or opportunistic infections in humans and animals (Zakaria, 2024). In the context of agricultural crops like apples, understanding the biology and ecology of *A. flavus* and *A. fumigatus* is crucial for assessing their potential impact on fruit health, post-harvest quality, and worker safety. Both fungi primarily play a role in the decomposition of organic matter but possess specific characteristics that enable them to cause various issues when they come into contact with crops, particularly in warm, moist environments (Hakiki and Susetya, 2021; Nji et al., 2023).

A. fumigatus is not typically considered a major pathogen in apple trees, making its presence in the apple orchards of Timor Island an unusual finding. *A. flavus*, on the other hand, is a well-known filamentous fungus famous for producing aflatoxins, potent mycotoxins that can contaminate agricultural products (Kumar et al., 2021). The fungus produces a variety of extracellular enzymes, including proteases (Farnell et al., 2012), lipases (Mehta et al., 2020), and amylases (Tiarsa et al., 2022), which break down plant tissues and facilitate fungal colonization. Ecologically, *A. flavus* thrives in warm temperatures ranging from 25°C to 35°C and in high humidity, making apples in warm climates or poorly ventilated storage areas particularly vulnerable to fungal contamination (Anggreini et al., 2019). It is commonly found in soil or on decaying plant material, with transmission occurring through airborne spores (Latgé and Chamilos, 2019).

Similarly, *A. flavus* is more commonly associated with producing aflatoxins in stored agricultural commodities rather than causing direct infections in live apple trees (Hocking, 2006). The role of these species as plant pathogens is likely opportunistic, potentially exacerbated by environmental stressors such as drought, nutrient deficiencies, or high temperatures (Nji et al., 2023).

This raises questions about the environmental conditions in the region that may have contributed to the atypical pathogenicity of *Aspergillus* species. It is plausible that stress factors, including climate extremes or poor soil health, may have predisposed the apple trees to infections by opportunistic fungi. Future research should investigate these environmental variables and determine whether *Aspergillus* spp. act as secondary invaders rather than primary pathogens.

Nevertheless, the findings of this study provide a solid

foundation for further research on the epidemiology, pathogenic mechanisms, and development of resistant cultivars to combat leaf spot disease in Soe apple orchards. The implementation of these research outcomes could improve apple production and enhance the livelihoods of farmers on Timor Island.

CONCLUSION

Three distinct fungal species were found to primarily infect apple trees on Timor Island, specifically in the five districts of Pubasu, Tunua, Tubuhue, Tobu, and Binaus. These species were *Fusarium solani*, *Aspergillus fumigatus*, and *A. flavus*. This study provides molecular identification and characterization of the major pathogens responsible for leaf spot disease in Soe apple cultivars on Timor Island. The identification of *A. flavus*, *A. fumigatus*, and *F. solani* as the main pathogens associated with leaf spot disease underscores the need for effective pest and disease management strategies to mitigate their detrimental effects on apple orchards.

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AUTHORS' CONTRIBUTIONS

BYT, RWS, SV, and WDS were involved in sample collection, isolation, and purification of pathogenic fungi; FP, DDM, and ESH participated in DNA extraction and molecular identification; all the authors approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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