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First record of *Aspergillus hongkongensis* C.C. Tsang et al. (Eurotiales, Ascomycota) in South America and the third report worldwide

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Abstract

The genus *Aspergillus* P. Micheli is among the most abundant fungi worldwide. Numerous species belonging to this genus have been reported to contaminate food and cause infections. We isolated *Aspergillus hongkongensis* C.C. Tsang et al. from eggplant flour sold in Recife, Brazil. The isolate was identified using morphological and molecular analyses (beta-tubulin and calmodulin genes). This is the first record of *A. hongkongensis* in South America and the third report worldwide. Our study contributes to a better understanding of the phenotypic variations in this species, as well as the geographic distribution of *Aspergillus* species.

Keywords

Eggplant flour, food contaminants, *Nidulantes*, taxonomy

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Introduction

Aspergillus P. Micheli is a genus of cosmopolitan fungi belonging to the family Aspergillaceae and currently contains approximately 446 species (Houbraken et al. 2020). The constant emergence of new strains within this genus reveals its high biodiversity. Owing to their varied characteristics, the strains of this genus can grow on diverse substrates, occurring in nature as endophytes, saprophytes, parasites, food contaminants, and human pathogens (Houbraken et al. 2014, 2020; Frisvad and Larsen 2015; Wang and Zhuang 2022).

According to Houbraken et al. (2020), *Aspergillus* is subdivided into six subgenera and 27 sections, among which the section *Nidulantes* currently contains seven series (series *Aurantiobrunnei*, *Multicolores*, *Nidulantes*, *Speluncei*, *Stellati*, *Unguium*, and *Versicolores*). This section includes species with varying characteristics; some

can cause superficial and systemic infections and produce mycotoxins, whereas others are air and food contaminants (Klich 2009; Hubka et al. 2012; Houbraken et al. 2014; Visagie et al. 2014b; Chen et al. 2015; Frisvad and Larsen 2015; Tsang et al. 2016). When studying the cause of onychomycosis in human fingernails, Tsang et al. (2016) isolated and described *Aspergillus hongkongensis* C.C. Tsang et al. as a novel etiological agent of this mycosis. This species currently belongs to the series *Versicolores*, and it was also recently isolated from bat guano-contaminated soil samples from the Gcwihaba Cave in the Okavango basin, Botswana (Visagie et al. 2021).

Flours are generally considered microbiologically safe owing to their low water activity that prevents the growth of microorganisms and ensures higher-quality products (Berghofer et al. 2003; Soquetta et al. 2016; Láscaris et al. 2020). Although there are few reported cases of food poisoning caused by contaminated flour, some microbiological studies conducted on different flours types have shown that the fungal community is diverse. Most of the identified species are the primary mycotoxin-producing contaminants belonging to the genera *Aspergillus* and *Penicillium* Link and possess a xerophilic character (Weidenbörner et al. 2000; Gashgari et al. 2010; Lima 2019; Sousa et al. 2021).

The different sources and processes from which fungal contamination can occur in flours include raw material harvesting, cleaning, flour manufacturing, packaging, transport, and storage. These conditions can be avoided if the raw material is stored in accordance with the recommended standards for food processing until the final product is sold (Souza et al. 2004; Silva et al. 2017; Lima et al. 2020).

During a study on the microbiological and physicochemical composition of eggplant flour in Recife, Brazil, a strain of *A. hongkongensis* was isolated. This species was previously reported to cause onychomycosis in human nails in Hong Kong (Tsang et al. 2016). Here, we present a detailed description and illustration of *A. hongkongensis* and record the first occurrence of this species in South America and the third report worldwide.

Methods

Isolation and purification. Eggplant flour samples sold in bulk were obtained from a specialized store in the metropolitan region of Recife, Brazil, during September to November 2017. The samples were collected from three different batches and stored in sterile plastic bags. Each sample was analyzed within 24 h of acquisition. The fungi were isolated according to the method described by Alhadas et al. (2004). Samples were initially subjected to surface sterilization with 0.2% sodium hypochlorite solution for 2 min, rinsed thrice with sterile distilled water, and then the excess water was dried with sterile filter paper. The packages were opened under aseptic conditions, and the dilution technique was used for plating. Briefly, 25 g of each sample was removed, and 225 mL of 0.1% peptone water (1:10) was added serially until 1:1000 dilution was reached. This mixture was then shaken, and 1 ml aliquots were spread on the surface of Petri dishes with dichloran rose bengal chloramphenicol base agar (DRBC), dichloran glycerol agar 18% (DG18), and malt salt agar (MSA) in triplicate (King et al. al. 1979; Hocking and Pitt 1980; Neves 2013). Plates were incubated for 10 days at 25 ± 1 °C. The obtained fungal colonies were purified and subcultured on potato dextrose agar (PDA) or malt extract (MEA) and incubated at 25 °C for 5–7 days for taxonomic characterization and identification.

Morphology. Morphological analyses were performed according to the recommendations of Barbosa et al. (2018). Briefly, each strain was inoculated at three timepoints in 9-cm plastic Petri dishes using a dense suspension of conidia on Czapek yeast extract agar (CYA), malt extract agar (MEA), oatmeal agar (OA), Czapek agar (CZ), CYA supplemented with 5% NaCl (CYAS), creatine agar (CREA), and yeast extract sucrose agar (YES). All media were prepared as described by Samson et al. (2010). To determine the culture characteristics (growth rate, colony texture, pigmentation, and exudates), plates were incubated in the dark at 25 °C for seven days. The color names and alphanumeric codes were provided as described by Rayner (1970). Microscopic observations of colonies grown on MEA were performed. Cultural and microscopic features were compared to previous descriptions by Tsang et al. (2016). The isolate was deposited in the URM culture collection (Micoteca URM Profa. Maria Auxiliadora Cavalcanti (WCDM 604) of the Federal University of Pernambuco (UFPE), Recife, Brazil (Barbosa et al. 2020).

DNA sequence analysis. Genomic DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's recommendations. The *BenA* (beta-tubulin) and *CaM* (calmodulin) regions were amplified using the primers and PCR conditions described by Visagie et al. (2014a). The PCR products were purified with exonuclease I and alkaline phosphatase enzymes contained in the Illustra ExoProStar 1-Step kit (GE Healthcare), according to the manufacturer's guidelines, and subsequently sent for sequencing with the same primers using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies, Carlsbad, CA, USA) on the Multiuser Sequencing and Gene Expression Platform of the Biological Sciences Center of UFPE.

Phylogenetic analyses. Nucleotide sequences were edited and assembled from both directions using the SeqMan program v. 10.0.1 and analyzed using the BLASTn program on the NCBI database platform. The sequence database of each strain was generated using sequences of type materials previously published and available from the NCBI. The sequences obtained in this study were aligned through the multiple alignments

using fast Fourier transform program (Katoh et al. 2005), and the alignments were manually optimized using the Molecular Evolutionary Genetics Analysis (MEGA 7) software (Tamura et al. 2013). Nucleotide substitution models were determined using jModelTest v. 2.1.7 (Posada 2008). Maximum likelihood (ML) phylogenetic analyses were performed using RAxML-VI-HPC v. 7.0.3 (Stamatakis 2006) and Bayesian inference (BI) analysis in MrBayes v. 3.2.1 (Ronquist et al. 2012). Phylogenetic trees were visualized using FigTree v. 1.1.2 (Rambaut 2009) and edited in Adobe Illustrator v. CS5.1. For combined analyses, individual alignments were concatenated using the Mesquite v. 3.04 software (Maddison and Maddison 2016).

Results

Aspergillus hongkongensis **Tsang et al.** Diagnostic Microbiology and Infectious Disease 84(2): 130 (Tsang et al. 2016). [MB810279]
Figure 1A–D

New record. BRAZIL – **Pernambuco** • Recife city; 08° 03′06″S, 034°57′10″W; 15.IX.2017; J.M.S. Lima leg.; habitat: eggplant flour; GenBank: *BenA:* OU898275, *CaM:* OU898274; URM 8457.

Identification. Colony characteristics: CYA 25 °C, 7 days: 19–20 cm in diameter, colonies plane, slightly raised at the center; margins entire, regular; mycelia white; sclerotia absent; texture velvety; degree of sporulation poor; conidia *en masse* indeterminate; soluble pigments absent; exudates absent; reverse pigmentation salmon (41) to cinnamon (62). MEA 25 °C, 7 days: 20–25 cm in diameter, colonies plane, umbonate; margins entire, regular; mycelia white; sclerotia absent; texture velvety; degree of sporulation moderate at the center; conidia *en masse* greenish olivaceus (90); soluble pigments absent; exudates absent; reverse pigmentation pale luteus (11). OA 25 °C, 7 days: 20–23 cm in diameter, colonies plane, slightly raised at the center; margins entire, regular; mycelia white; sclerotia absent; texture velvety; degree of sporulation strong at the center; conidia *en masse* dull green (70); soluble pigments absent; exudates absent; reverse pigmentation quietly primrose (66). YES 25 °C, 7 days: 32–35 cm in diameter, colonies plane, raised at the center; margins entire, regular; mycelia white; sclerotia absent; texture velvety; degree of sporulation poorly, spread near the center; conidia *en masse* quietly buff (45), soluble pigments absent; exudates absent; reverse pigmentation ochreous (44) became strong at sienna (8). CZ 25 °C, 7 days: 20–25 cm in diameter, colonies plane with radial grooves; raised at the center; margins entire, regular; mycelia white; sclerotia absent; texture velvety; degree of sporulation moderate at the center; conidia *en masse* white to smoke grey (105); soluble pigments absent; exudate sepia (63) droplets; reverse pigmentation salmon (41) to saffron (10) in the center. CYAS 25 °C, 7 days: 19–20 cm in diameter, colonies plane; raised at the center; margins entire; mycelia white; texture velvety; sclerotia absent; degree of sporulation poor, conidia *en masse* indeterminate; soluble pigments absent; exudates absent; reverse salmon (41). CREA 25 °C, 7 days: 20–23 cm in diameter, colonies plane, slightly raised at the center; margins entire, regular; mycelia white; acid production absent. Micromorphology: conidiophores biseriate. Stipes hyaline, 150–540 × 2.5–4.0 µm, smooth-walled, rarely septate, foot cell symmetric, sometimes asymmetric. Conidial heads columnar, sometimes radiate; vesicles 7–17 × 6–9.5 µm, oval to clavate. Metulae 4.0–6.0 × 1.5–2 µm, wedge-shaped. Phialides on each metula, 4.5–6.0 × 1.5–2.5 µm, cylindrical. Conidia 2–3 µm in diameter, globose, rough-walled, and hyaline. No accessory conidia, ascomata, ascospores, or Hülle cells were observed.

Phylogenetic analyses of *A. hongkongensis* and related species were performed for the *BenA* and *CaM* genes, as well as the concatenation of both. Results showed that the *A. hongkongensis* URM 8457 sequence was placed with high support values in the same clade as the *A. hongkongensis* HKU49^{T} sequence (Fig. 2). BLASTn analysis of the *BenA* and *CaM* region/gene sequences showed 98.80%, and 100.00% identity, respectively, between URM 8457 and the holotype.

Distribution. China (Hong Kong), Botswana and Brazil (Pernambuco, Recife). Figure 3.

Discussion

This study provides a new record of *A. hongkongensis* in Brazil, extending their geographic distribution. The species belongs to the phylum Ascomycota, class Eurotiomycetes, order Eurotiales, family Aspergillaceae, and genus *Aspergillus*, which comprises at least 446 species. With advancements in molecular phylogeny, numerous rearrangements have been enacted in the classification of this genus, and its species are currently distributed across six subgenera, approximately 27 sections, and 75 series (Houbraken et al. 2020). Several species have diverse properties, and include food-spoilage organisms (e.g., *A. proliferans* G. Sm.), mycotoxin producers (e.g., *A. flavus* Link), and human pathogens (e.g., *A. fumigatus* Fresen. and *A. flavus*) (Sugui et al. 2014; Greco et al. 2015; Frisvad et al. 2019). Members of *Aspergillus* are generally referred to as osmo-, xero-, or halotolerant. They are distributed worldwide, grow on different substrates under different conditions, and are abundantly found in semi-dry foods (Pitt and Hocking 2009). *Aspergillus hongkongensis* is included in the section *Nidulantes*, which has 16 species, all of which are anamorphic, and the vast majority are reported to cause infections/health problems owing to the production of mycotoxins (Rank et al. 2011; Jurjevic et al. 2012; Hubka et al. 2016; Tsang et al. 2016; Chrenková et al. 2018).

Species belonging to this section are often reported as food contaminants. *Aspergillus sydowii* (Bainier &

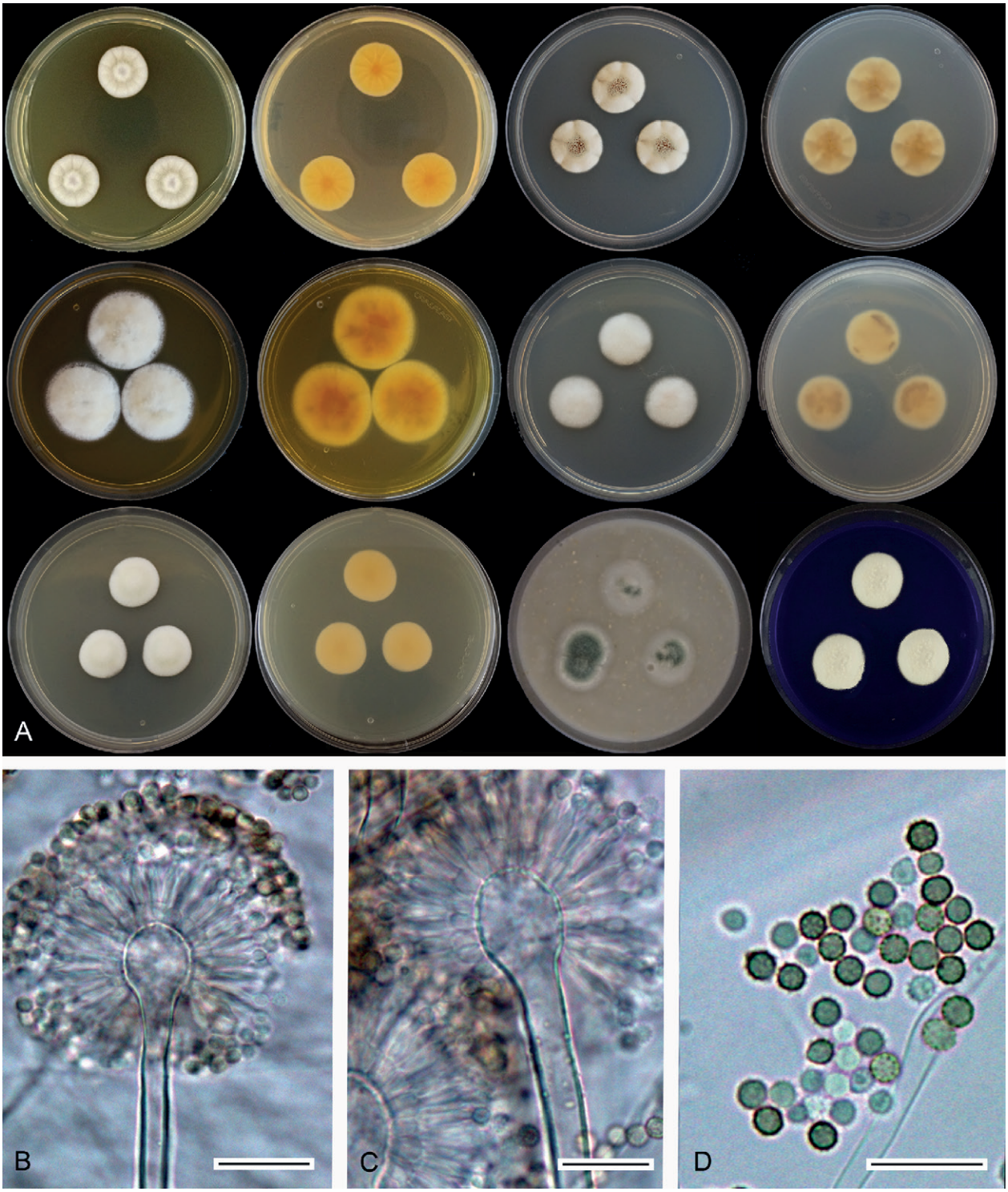

Figure 1. *Aspergillus hongkongensis* URM 8457. **A.** Colonies from left to right (top row) grown on MEA plate and its reverse, CZ plate and its reverse, YES plate and its reverse, CYA plate and its reverse, CYAS plate and its reverse, OA, and CREA. **B, C.** Details of conidiophores with vesicles and phialides. **D.** Conidia. Scale bars: B, C = 10 µm; D = 50 µm.

Sartory) Thom & Church has been found in samples of black pepper and cassava flour (Silva et al. 2012; Ekpakpale et al. 2021), and *A. versicolor* (Vuill.) Tirab. in samples of carob and wheat flours (Cabañas et al. 2008; Plavšić et al. 2016; Mom et al. 2020). According to Chen et al. (2016), many *Aspergillus* species belonging to the section *Nidulantes* produce aflatoxins. This is the first recorded report of *A. hongkongensis* in food from Latin America and the third report worldwide. In our molecular data, the new record clustered into a well-supported clade (1.00bl, 100%), with the type species being the only known strain to date. Morphologically, our lineage (URM 8457) was consistent with the original description (Tsang et al. 2016); however, some phenotypic variations were observed between the description of the ex-type lineage [HKU49 (T)] and our lineage (URM 8457). These variations include differences in growth rates at 25 °C on CYA (26 mm vs. 20 mm), MEA (22 mm vs. 20–25

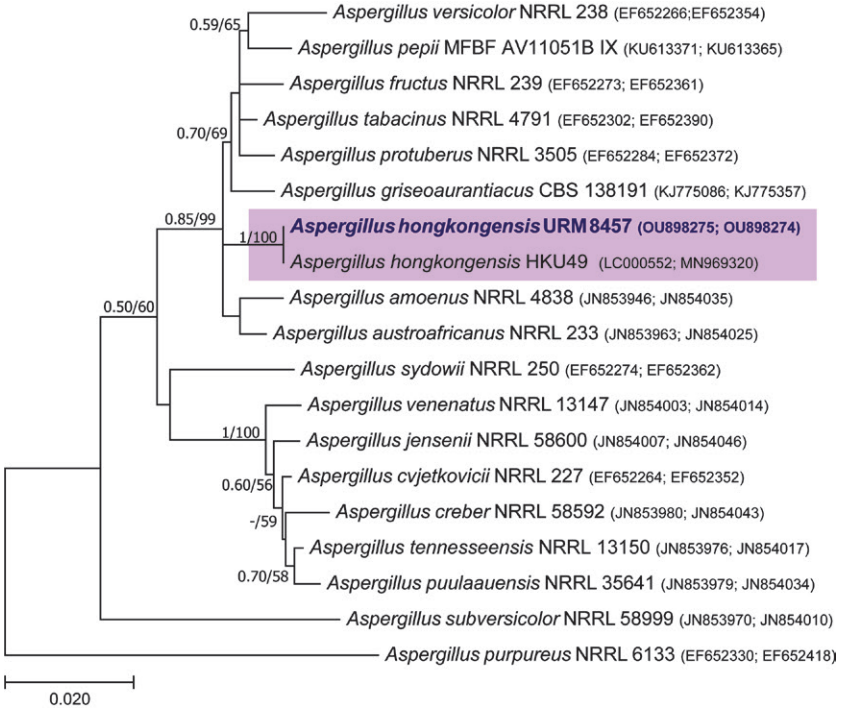

Figure 2. Phylogenetic tree of *Aspergillus* series *Versicolores* of section *Nidulantes* and *Aspergillus hongkongensis* URM 8457, constructed using combined *BenA* and *CaM* genes sequences. *Aspergillus purpureus* NRRL 6133 was used as outgroup. The sequence obtained in this study is in bold. Bayesian inference (BI), posterior probabilities (pp) values, and bootstrap (bs) values are labelled at the nodes. Values less than 0.50 pp and 50% bootstrap support are not shown.

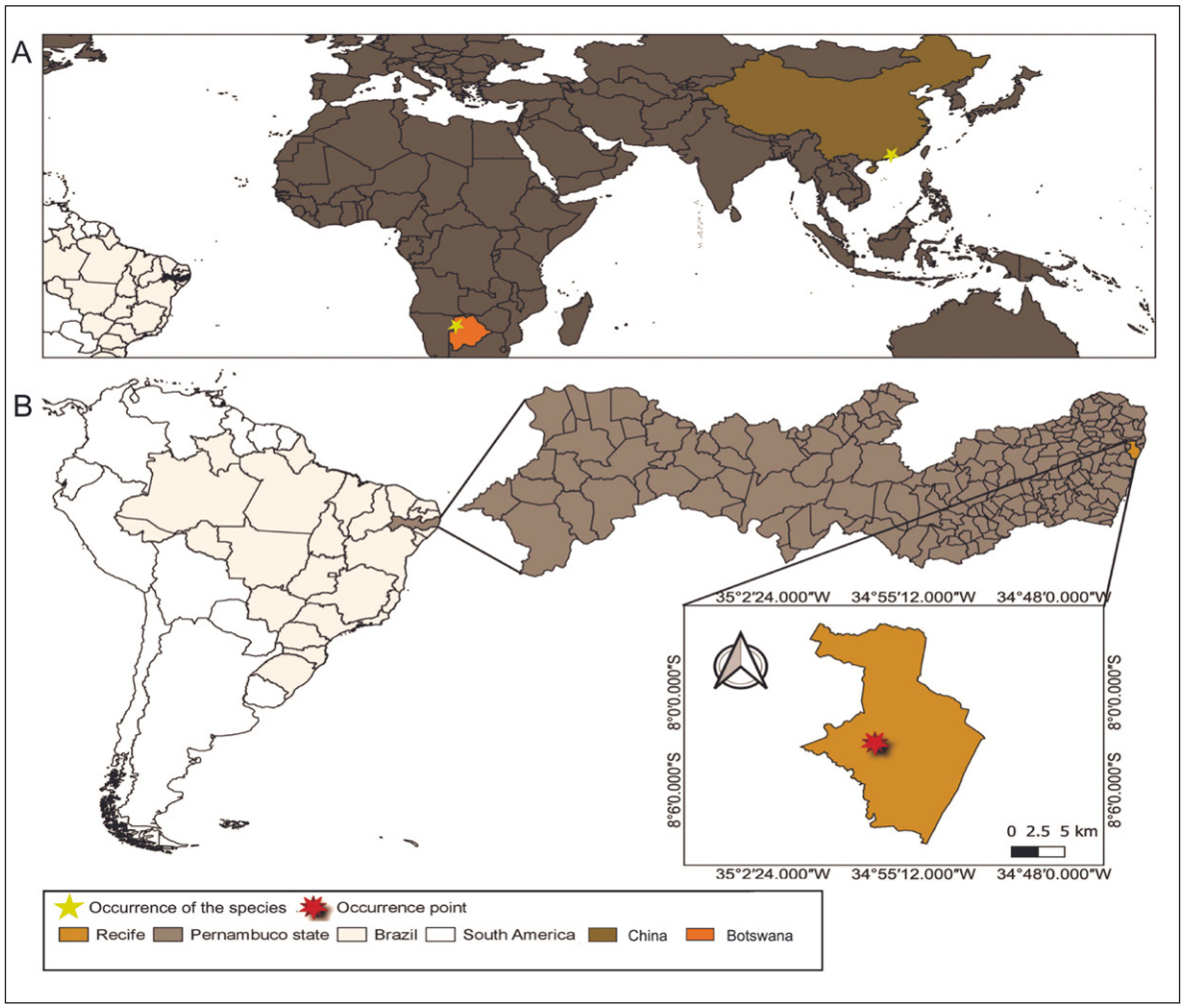

Figure 3. Map depicting the locations of occurrence (known and new record) of *Aspergillus hongkongensis*.

mm), and CZ (18 mm vs. 20–25 mm). In addition, some differences in colony color and the presence of soluble pigments in CZ culture were observed. *Aspergillus hongkongensis* was first reported from clinical samples as the causative organism of onychomycosis (Tsang et al. 2016). Strains isolated from clinical samples typically have phenotypes that are different from those of environmental strains. Our record was obtained from food with low water activity, and more strains (from various substrates) are needed to show whether these differences are related to niche differentiation.

We report the first occurrence of *A. hongkongensis* in South America, its first report in a food product, and the third recorded occurrence worldwide. Our report on this species isolated from eggplant flour sold in Recife-Pernambuco, Brazil, contributes to the knowledge of the geographic distribution of the Eurotialian fungi.

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Authors' Contributions

Conceptualization: JMSL, RC. Data curation: JMSL. Formal analysis: RB, DXL. Funding acquisition: CMSM. Investigation: JMSL, RC. Methodology: JMSL, LVS. Resources: CMSM. Supervision: CMSM, RC, JMSL. Visualization: JMSL, RB. Project administration: CMSM, JMSL. Software: RB, DXL. Validation: JMSL, CMSM. Writing – original draft: JMLS, RNB, DXL. Writing - review and editing: JMSL, CMSM, RC, DXL, RNB, LVS.

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