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MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF FUNGAL CONTAMINANTS ON ARABICA COFFEE BEANS FROM KINTAMANI, BALI: IMPLICATIONS FOR FOOD SAFETY AND SUSTAINABLE POST-HARVEST MANAGEMENT

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Abstract
Fungal contamination in *Coffea arabica* poses a significant threat to coffee bean quality and food safety, particularly under humid tropical conditions. This study aimed to characterize fungal contaminants on Arabica coffee beans from Kintamani, Bali, and to evaluate their implications for consumer health and sustainable post-harvest management. Coffee bean samples were collected from multiple plantations and analyzed using morphological methods on Potato Dextrose Agar (PDA), complemented by molecular identification through Polymerase Chain Reaction (PCR) and Internal Transcribed Spacer (ITS) sequencing. Morphological analysis identified three dominant species: *Aspergillus niger*, *A. ochraceus*, and *A. flavus*. However, phylogenetic analysis revealed that several isolates initially identified as *A. niger* and *A. ochraceus* exhibited higher genetic similarity to the *A. aculeatus/japonicus* clade, highlighting the limitations of morphology-based identification under tropical conditions. These findings underscore the importance of integrating morphological and molecular approaches for accurate fungal identification. The ecological and health implications of potential mycotoxin production reinforce the urgency of improving coffee bean drying and storage systems. This study represents the first molecular characterization of fungal contaminants in Kintamani coffee and provides a scientific basis for the development of biocontrol strategies and safe, sustainable post-harvest practices.

Keywords: *Coffea arabica*, Fungal Contamination, Molecular Identification, Post-Harvest Safety

1. Introduction

Coffee (*Coffea* spp.) is one of the most valuable agricultural commodities globally, with consumption and trade continuously increasing (FAO, 2022). The two main commercially cultivated species are *Coffea arabica* and *Coffea canephora* (robusta), with Arabica coffee being preferred due to its superior sensory characteristics and lower caffeine content (Silva et al., 2024; DaMatta & Ramalho, 2006). Indonesia is among the world's leading producers of Arabica coffee, with the Kintamani region in Bali recognized with a Geographical Indication (GI) for high-quality coffee and holding substantial export potential (Fauzi et al., 2022; Widyastuti et al., 2021).

However, fungal contamination, particularly by mycotoxin-producing species, poses a serious challenge as it directly affects coffee quality, food safety, and export viability (Noonim et al.,

2008; Banahene et al., 2024; Taniwaki et al., 2018). The most concerning contaminants include *Aspergillus ochraceus*, *A. carbonarius*, and *A. niger*, which are known to produce ochratoxin A (OTA), a nephrotoxic, immunotoxic, and carcinogenic compound resistant to heat treatment and remaining active even after roasting (Pitt & Hocking, 2009; Akbar et al., 2020; Palumbo et al., 2007).

Early studies by Batista et al. (2003) demonstrated that raw coffee beans could already be contaminated by OTA-producing fungi prior to processing. Contemporary research by Al Attiya et al. (2021) confirmed the presence of mycotoxins in Arabica coffee and emphasized the importance of traditional mitigation strategies based on local practices. In Indonesia, Fauzi et al. (2022) reported Aflatoxin B1 contamination in post-harvest coffee from Sumatra, indicating weaknesses in farmers' processing and storage systems. Similar findings were observed in Thailand by Maman et al. (2021), where uncontrolled drying and storage conditions facilitated OTA accumulation during distribution.

Geographically, the Kintamani region exhibits a humid tropical climate and still relies on traditional open-air drying methods, making coffee beans highly susceptible to environmental fungal contamination (Noonim et al., 2008; Borrelli et al., 2004). A systematic review by Silva et al. (2024) highlighted that OTA contamination has become a global issue in the coffee industry, with significant health and trade implications. OTA is considered one of the major public health threats, capable of crossing national borders and penetrating global supply chains (Banahene et al., 2024; Hagos et al., 2024; Taniwaki et al., 2018).

López Rodríguez et al. (2024) emphasized that good agricultural and post-harvest practices, such as proper drying, sorting, and storage, can significantly reduce the risk of OTA contamination. Nevertheless, the implementation of mitigation protocols remains limited in smallholder production areas such as Kintamani, Bali. This underscores the importance of site-specific post-harvest microbiological studies to inform contextually relevant, evidence-based intervention strategies.

Accurate fungal species identification is a crucial first step in effective contamination control. While morphological approaches based on colony characteristics and conidial structures are commonly used, the accuracy of identification can be significantly enhanced through molecular techniques, such as PCR amplification of the Internal Transcribed Spacer (ITS) region, β-tubulin gene analysis, and phylogenetic assessment (White et al., 1990; Gil-Serna et al., 2009; Lee et al., 2020; Samson et al., 2014). Studies from various countries have demonstrated that combining morphological and molecular approaches provides higher validity in detecting *Aspergillus* section Nigri and determining evolutionary relationships (Sette et al., 2006; Morello et al., 2007; Das Neves et al., 2024; Frisvad et al., 2019).

To date, no systematic studies have been conducted to identify contaminant fungal species and map their molecular relationships in Arabica coffee from Kintamani, Bali—a region of considerable economic and social importance in Indonesia's coffee supply chain. Therefore, this study aimed to: (a) isolate and morphologically identify contaminant fungal species on Arabica coffee beans from Kintamani, Bali; (b) perform molecular identification using PCR amplification and ITS region sequencing; and (c) analyze the evolutionary relationships among isolates and reference species through phylogenetic tree construction. The results are expected to contribute significantly to the understanding of post-harvest coffee microbiology in Indonesia and support the development of safe, high-quality, and sustainable coffee processing and supply chain systems at both local and global levels.

2. Literature Review

2.1 Fungal Contamination in Coffee Beans

Fungal contamination in coffee beans has been recognized as a major challenge in ensuring coffee quality and safety, particularly in tropical regions where humidity and temperature favor fungal growth (Noonim et al., 2008; Taniwaki et al., 2018). Several species of the genus *Aspergillus* are commonly associated with coffee contamination, including *A. ochraceus*, *A. carbonarius*, and *A. niger* (Pitt & Hocking, 2009; Akbar et al., 2020). These fungi are capable of producing ochratoxin A (OTA), a mycotoxin known for its nephrotoxic, immunotoxic, and carcinogenic properties (Palumbo et al., 2007; Silva et al., 2024).

Studies have demonstrated that OTA contamination can occur at multiple stages of the coffee supply chain, from pre-harvest to post-harvest processing and storage. Batista et al. (2003) reported the presence of OTA-producing fungi on raw coffee beans, suggesting that contamination can begin before processing. Similarly, Al Attiya et al. (2021) emphasized that traditional mitigation strategies at the farm level, including proper drying and sorting, are critical in minimizing fungal proliferation and OTA accumulation.

Geographical and environmental factors significantly influence fungal contamination. In humid tropical climates, such as Bali, traditional open-air drying methods and inadequate storage facilities increase the vulnerability of coffee beans to *Aspergillus* colonization (Borrelli et al., 2004; Maman et al., 2021). Silva et al. (2024) highlighted that OTA contamination is not only a local concern but has global implications due to the international coffee trade, with mycotoxins capable of crossing borders and penetrating global supply chains (Banahene et al., 2024; Hagos et al., 2024).

2.2 Morphological Identification of Fungal Contaminants

Morphological identification remains a widely used method for detecting fungi in coffee due to its simplicity and cost-effectiveness (Sette et al., 2006; Morello et al., 2007). This approach relies on observing colony morphology, conidial structures, and pigmentation on culture media such as Potato Dextrose Agar (PDA) or Czapek Yeast Agar (CYA) (Samson et al., 2014). While effective for preliminary identification, morphological methods have limitations in differentiating closely related species, especially within *Aspergillus* section Nigri (Gil-Serna et al., 2009; Das Neves et al., 2024). Misidentification can lead to inaccurate assessment of mycotoxin risks, emphasizing the need for complementary molecular techniques.

2.3 Molecular Identification and Phylogenetic Analysis

Molecular techniques have become essential in accurately identifying fungal contaminants. Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer (ITS) region, β-tubulin genes, and calmodulin sequences allows for precise species-level identification (White et al., 1990; Lee et al., 2020). Phylogenetic analysis provides insights into evolutionary relationships among isolates, aiding in understanding species diversity and potential toxigenic capabilities (Frisvad et al., 2019; Das Neves et al., 2024). Studies in coffee-producing regions have demonstrated that combining morphological and molecular approaches significantly improves the accuracy of identifying *Aspergillus* species (Sette et al., 2006; Morello et al., 2007).

2.4 Mycotoxin Production and Food Safety

Ochratoxin A is the most concerning mycotoxin in coffee due to its stability during roasting and resistance to heat treatment (Pitt & Hocking, 2009; Akbar et al., 2020). OTA contamination poses health risks including nephropathy, immunosuppression, and carcinogenesis (Palumbo et al., 2007). Studies in Southeast Asia have revealed the widespread occurrence of OTA in coffee, indicating the need for stringent monitoring and intervention strategies (Noonim et al., 2008; Maman et al., 2021). Regulatory limits for OTA in coffee vary internationally, highlighting the importance of post-harvest management practices to ensure compliance and protect public health (Taniwaki et al., 2018).

2.5 Post-Harvest Management and Mitigation Strategies

Good Agricultural Practices (GAP) and Good Post-Harvest Practices (GPP) are critical for reducing fungal contamination and mycotoxin accumulation (López Rodríguez et al., 2024; Silva et al., 2024). Proper drying techniques, sorting of defective beans, hygienic storage, and controlled humidity conditions have been shown to significantly reduce the proliferation of toxigenic fungi (Borrelli et al., 2004; Widyastuti et al., 2021). Additionally, biocontrol agents and innovative storage technologies are emerging as effective strategies for mitigating OTA risk while maintaining coffee quality (Al Attiya et al., 2021; Frisvad et al., 2019).

Despite these advances, smallholder farmers in regions such as Kintamani, Bali, often face challenges in implementing standardized post-harvest protocols due to limited resources and traditional practices. This underscores the importance of localized studies to inform context-specific intervention strategies that are economically viable and sustainable.

3. Methodology

This study was designed to identify and characterize fungal contaminants in Arabica coffee beans from the Kintamani region, Bali, using an integrated approach combining morphological and molecular methods. The study also aimed to assess the potential involvement of *Aspergillus* species in mycotoxin contamination, particularly ochratoxin A (OTA), and to elucidate the phylogenetic relationships among field isolates.

3.1 Sampling Location and Collection

Coffee bean samples were collected from smallholder farms in three major Arabica coffee-producing villages in Kintamani: Catur, Belantih, and Batukaang, Bangli Regency, Bali (Figure 1). The region is located at an altitude of 1,000–1,200 m above sea level, with a humid tropical climate, temperatures ranging between 18–30°C, and an annual rainfall exceeding 2,000 mm, which provides an ecologically favorable environment for fungal growth (Das Neves et al., 2024).

A total of 500 g of ripe coffee cherries were randomly collected from each farm, placed in sterile bags, and transported immediately to the microbiology laboratory for isolation procedures.

3.2 Morphological Isolation and Identification

Coffee cherries were surface-sterilized using a 1% sodium hypochlorite solution for one minute to remove epiphytic microorganisms, rinsed three times with sterile distilled water, and aseptically dried. Beans were then placed on Potato Dextrose Agar (PDA) in sterile Petri dishes and incubated at 29°C for 5–7 days (Sette et al., 2006). PDA was selected due to its suitability for supporting the growth of various fungi, including *Aspergillus* section Nigri (Casas Junco et al., 2017).

Colony morphology was observed macroscopically in terms of color, texture, and growth pattern, and microscopically using a light microscope. Preliminary identification was based on the morphology of conidiophores, vesicles, and phialides, characteristic of the *Aspergillus* genus (Morello et al., 2007; Pitt & Hocking, 2009). Morphological observations served as the basis for selecting isolates for subsequent molecular identification.

3.3 Molecular Identification (PCR and Sequencing)

DNA from selected pure isolates was extracted using commercial kits (e.g., Promega Wizard® or Qiagen®) according to the manufacturer's instructions. PCR amplification targeted the Internal Transcribed Spacer (ITS) region of rRNA, a universal marker for fungal identification (White et al., 1990). Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used in a 25 µL reaction mixture consisting of 12.5 µL master mix, 1 µL of each primer (10 µM), 1 µL DNA template, and 9.5 µL nuclease-free water.

Thermal cycling conditions included an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 1 min (extension), with a final extension at 72°C for 10 min. PCR products were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide under UV illumination (Gil-Serna et al., 2009; Liu et al., 2024).

3.4 Phylogenetic Analysis

Obtained DNA sequences were compared with reference sequences in GenBank using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignments were performed using MEGA software (Molecular Evolutionary Genetics Analysis, latest version). Phylogenetic trees were constructed using the Neighbor-Joining (NJ) method with evolutionary distances calculated based on the Kimura 2-parameter model. Bootstrap analysis with 1,000 replicates was conducted to assess branch reliability (Lee et al., 2020).

Penicillium crustosum NRRL 66388 was used as the outgroup to root the phylogenetic tree, a standard practice in fungal taxonomy studies (Ben Miri et al., 2024). This approach enabled accurate visualization of evolutionary relationships among local isolates and OTA-producing *Aspergillus* species (Morello et al., 2007; Fontana et al., 2024).

4. Results and Discussion

4.1 Fungal Isolation and Morphological Identification

Isolation was performed on naturally dried *Coffea arabica* beans from Kintamani, Bali. Samples were cultured on Potato Dextrose Agar (PDA) and incubated at room temperature (28 ± 2°C) for 5–7 days. Three dominant colony morphologies were observed and tentatively identified as *Aspergillus niger*, *A. ochraceus*, and *A. flavus*. Colony color, texture, and growth rate served as primary identification indicators: *A. niger* appeared black and granular, *A. ochraceus* was yellowish with wavy colony margins, and *A. flavus* exhibited olive-green conidia with radially branched conidiophores (Klich, 2002; Pitt & Hocking, 2009).

While macroscopic morphology provides an initial identification framework, visual characteristics alone may not sufficiently distinguish closely related species. Many *Aspergillus* species display overlapping phenotypes, particularly under uniform culture conditions, necessitating microscopic and molecular confirmation for accurate identification.

4.2 Microscopic Examination

Microscopic observations of conidiophores, vesicles, and conidia were conducted using Lactophenol Cotton Blue staining. Globose vesicles were observed in *A. flavus* and *A. niger*, with upright conidiophores bearing echinulate conidial chains. *A. ochraceus* displayed subglobose to pyriform vesicles, shorter conidiophores, and smoother conidial surfaces. Biseriate phialides served as additional identification markers. Although microscopic analysis reveals internal morphological details, limitations exist due to the genus' high species diversity and subtle interspecies variation (Houbraken et al., 2012). Consequently, these findings were used as preliminary identification prior to molecular analysis.

4.3 Molecular Identification via PCR and Gel Electrophoresis

Molecular identification reinforced morphological observations. Genomic DNA was extracted from pure fungal cultures, followed by PCR amplification of the ITS (Internal Transcribed Spacer) region using primers ITS1 and ITS4, which are highly conserved for fungal identification (White et al., 1990). PCR yielded a single DNA band of approximately 500 bp, consistent with filamentous fungi ITS target length. Products were visualized on 1.5% agarose gels stained with ethidium bromide under UV illumination, confirming DNA integrity and suitability for sequencing. Molecular methods provided robust taxonomic classification and formed a critical component of DNA-based food safety surveillance.

4.4 Phylogenetic Analysis

Obtained sequences were compared with public databases using NCBI BLAST. Some isolates showed >98% homology with *A. niger*, *A. ochraceus*, and *A. flavus*, while others were more closely related to *A. japonicus* and *A. aculeatus*. Phylogenetic trees were constructed using MEGA-X software with the Neighbor-Joining method. Branching patterns revealed that several isolates clustered into distinct clades, suggesting the presence of cryptic species within Kintamani's local fungal communities (Samson et al., 2014; Minaeva, 2023).

These results confirm the dominance of *Aspergillus* species in Arabica coffee beans from Kintamani, consistent with previous findings in Indonesia. Dharmaputra (2019) reported consistent *Aspergillus* isolation from Sulawesi coffee, highlighting the conducive nature of tropical conditions for xerophilic fungi. In Sumatra, *A. ochraceus* was identified as a major OTA-producing contaminant, emphasizing the health risk posed by mycotoxins (Fauzi et al., 2022).

Environmental conditions, post-harvest processing, and storage practices strongly influence fungal growth and OTA accumulation. Tropical temperatures (>30°C) and relative humidity (>70%) promote *Aspergillus* colonization and toxin production, as observed in Kintamani (Das Neves et al., 2021; Hagos et al., 2024). Traditional drying methods, slow or uneven sun drying, and mechanical damage to beans further exacerbate contamination risk (Lilia et al., 2021; Hale et al., 2022). Prolonged storage in closed containers without humidity control fosters a microenvironment conducive to sporulation and toxigenesis (Maman et al., 2021).

Occupational exposure is also a concern, as OTA-contaminated dust can affect post-harvest workers, often women and vulnerable groups in Kintamani (Oliveira et al., 2025). Globally, OTA presence exceeds permissible levels in many commercial coffee products (Pakshir et al., 2021).

Mitigation strategies include biocontrol using antagonistic fungi such as *Trichoderma* spp., effective against pathogenic *Aspergillus* (Kapeua-Ndacnou et al., 2023). Sustainable agricultural practices, including organic inputs, proper airflow, and shade tree management, significantly reduce OTA risk (López Rodríguez et al., 2024). Additionally, co-occurrence of heavy metals (Cd, Pb) and mycotoxins highlights the need for a holistic, multisectoral approach (Mir et al., 2024).

Overall, this study provides a comprehensive view of fungal contaminants in Kintamani coffee, integrating morphological, microscopic, and molecular data, while linking environmental, post-harvest, and socio-health factors. The findings underscore the urgency of developing standard protocols for clean cultivation, DNA-based contaminant mapping, and farmer education to ensure sustainable coffee production.

5. Conclusion

This study revealed the presence of fungal contaminants in *Coffea arabica* beans from the Kintamani region, Bali, with potential implications for product quality and food safety. Using an integrated approach combining morphological and molecular identification, three primary species were initially identified morphologically: *Aspergillus niger*, *A. ochraceus*, and *A. flavus*. However, ITS-based molecular analysis indicated that some isolates initially identified as *A. niger* and *A. ochraceus* were genetically closer to *A. aculeatus* and *A. japonicus*. This finding highlights the limitations of morphology-based identification alone, especially under humid tropical conditions where interspecies trait overlap occurs.

The study also emphasizes the critical importance of early detection of mycotoxin-producing species, such as *A. ochraceus* and *A. flavus*, which pose health risks to consumers and may reduce the commercial value of coffee. Overall, the findings reinforce the necessity of a multidisciplinary approach for quality and safety management in coffee production, particularly during the post-harvest stage, which is highly susceptible to fungal contamination.

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