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Detection and identification of *Aspergillus aculeatinus* in corn seeds and milled products in Laguna, Philippines

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Abstract

Aspergillus flavus and *A. parasiticus* are among the most common fungal contaminants in corn seeds. In this paper, we reported *A. aculeatinus* as a fungal contaminant of corn seeds. The fungus was isolated from corn seeds and milled products, with highest incidence at 13% in pericarp. The fungus identity was elucidated through a combined morpho–cultural and molecular characterization, using the combined sequences of the partial internal transcribed spacer (ITS) rDNA, partial ß–tubulin (*BenA*), and calmodulin (*CaM*) gene regions. The representative *A. aculeatinus* isolates were also found to contain the aflatoxin biosynthesis genes *aflR* and *nor–1* in a polymerase chain reaction (PCR) assay, indicating the potential of these isolates to produce aflatoxin. However, no aflatoxin was detected in the ELISA assay and thus, suggesting that the presence of the genes does not always indicate the presence of the toxin. Mycotoxins have a major role in food safety and food security thus, report of this species is important as part of the corn mycobiota. The increase in knowledge on fungi contaminating food systems is essential when developing control measures. To our knowledge, this is the first record of *A. aculeatinus* as a fungal contaminant in corn seeds and milled products in the Philippines.

Keywords – Aflatoxin biosynthesis genes – *Aspergillus aculeatinus* – postharvest pathology

Introduction

Corn, also known as maize (*Zea mays* L.), is an important crop worldwide and a staple food, alongside rice, in the Philippines, with about 2.03 million metric tons produced annually (Philippine Statistics Authority 2023). It is consumed as grits as a substitute or alternate to rice and it can also be mixed with rice. At the Institute of Plant Breeding (IPB) of the University of the Philippines Los Baños, corn grits have been commercially sold and promoted together with the Department of Agriculture (DA) Corn Program where IPBVar6 is popularly used in grits production. The particular variety has a high protein level and generally has a low glycemic index. During the processing of corn grits, the endosperm is separated from the seed coat or pericarp and embryo. The remnants produce by–products such as *darak* (corn bran) and *binlid* (corn meal) which can be used as feeds for livestock. During storage of corn seeds and milled products,

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exposure to fungal contaminants that cause seed defects are very common both in the field and during postharvest operations. Fungal species such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. are the dominant taxa reported to produce mycotoxins (Balendres et al. 2019). Mycotoxins are a significant concern, especially in tropical regions, like the Philippines, due to environmental and storage conditions favorable for the growth and development of mycotoxigenic fungi (Anukul et al. 2013). They can be carcinogenic, teratogenic, tremorgenic, hemorrhagic, nephrogenic, and hepatogenic to both humans and animals (Ab Majid et al. 2015). The knowledge of fungi contaminating corn seeds and its milled products as well as the fungi's potential to produce mycotoxins is paramount to food safety. *Aspergillus* is a kind of cosmopolitan spore–forming microorganisms that can produce some of the world's most important mycotoxins. Abbot (2002) has listed more than 15 types of mycotoxins produced by *Aspergillus*. Among the mycotoxins, aflatoxins are the most carcinogenic causing an estimated 25% or more of the world's food crops to be destroyed annually (WHO 2018).

Analyzing DNA sequences has become a useful approach to identifying fungi (Cai et al. 2009). The fungal species are identified by comparison of a fungus' DNA sequence with that of other sequences available in sequence repositories or databases (e.g. GenBank). Among the commonly used gene regions are the internal transcribed spacer (ITS) and the *β–tubulin* gene (*BenA*) (Balajee et al. 2005; Hong et al. 2005). The ITS region is considered the universal barcoding gene of fungi and is also used for the initial identification of *Aspergillus*. In addition to ITS, the *β–tubulin* and calmodulin are recommended as secondary identification markers for *Aspergillus* species identification (Samson et al. 2014). Combining morphological characteristics and sequence data from these three genes can reliably identify *Aspergillus* isolates at the species level.

In a study seeking the contamination of *Aspergillus* species, a fungus with a different morphotype to *A. flavus* and *A. niger* (two commonly isolated fungal species) was isolated from corn seeds and milled products. A literature review revealed no information or report of this fungal morphotype in the Philippines (Balendres 2023). Hence, this study was aimed at identifying an *Aspergillus* sp. associated with corn products during corn grits milling operations using a combination of cultural, morphological, PCR–based detection, and DNA sequencing method. This study also assessed the contamination and distribution of this fungal species on different corn–milled products and determined the presence/absence of aflatoxin biosynthesis genes in this fungus.

Materials & Methods

Sample Collection and Fungal Isolation

IPBVar6 corn was harvested in 2019 from Calamba and Magdalena, Laguna, Philippines. Corn cobs were shelled, and corn kernels were milled using the two–roller portable mill fabricated for the Cereals Section. One kg of each corn kernel and milled products (cracked corn, grits, pericarp, *darak* (corn bran), and *binlid* (corn meal) were collected and brought to the laboratory for fungal isolation. A total of 270 randomly selected samples from each milled product were surface disinfected in 10% (v/v) sodium hypochlorite solution (Zonrox, Green Cross Inc., Philippines) for five minutes, rinsed three times in sterile distilled water, and air–dried in sterile tissue paper. Using flame–sterilized forceps, samples were transferred onto a potato dextrose agar (PDA) medium [250 g potato, 20 g PTC agar (Pronadisa, Germany), and 18 g dextrose in 1 L of distilled water]. Non–sterilized samples were also plated in the PDA medium. For each milled product/sample (e.g., kernels, grits, etc.), there were 30 plates and each plate contained nine replicate samples and, thus, a total of 270 technical replicates per milled product. Samples were incubated at room temperature for three days, and fungal incidence was recorded. Randomly collected fungal isolates were purified and maintained in a new PDA medium.

Cultural and Morphological Identification

Identification of the fungus was initially made using combined cultural and morphological characterization in PDA (Himedia Laboratories Inc., India) and malt extract agar (MEA, 20 g malt extract, and 15 g agar). Colony characteristics such as color on both obverse and reverse sides and radial growth (mm) were assessed seven days post–incubation (dpi). Spores were characterized through an agar block set–up. A square block of PDA was placed on a glass slide, and sides were inoculated with spores and mycelial fragments of the pathogen. A coverslip was then positioned on the block's top surface. The setup was moistened with sterile distilled water (in a Petri dish). After two dpi, the coverslip was mounted onto a new glass slide with a drop of cotton blue. Photomicrographs were taken and spore characteristics (conidia and conidiophore shape and size) of the fungi were assessed using a light microscope (Olympus CX22, Japan). A representative isolate showing similar morphotypes was selected for molecular characterization.

DNA Extraction

Fungal genomic DNA was extracted from a two–day–old fungus grown in potato dextrose broth using the procedure described by Liu et al. (2000). The mycelia were manually homogenized using a micro pestle with 500 µL of lysis buffer (400 mM Tris–HCl (pH 8.0); 60 mM EDTA (pH 8.0), 150 mM NaCl, 1% SDS) in 1.5 mL microcentrifuge tubes. The tubes were incubated at 25 °C for ten minutes. Then 150 µL of potassium acetate (pH 4.8) was added to the tube and subsequently vortexed and centrifuged (10,000 × g for 1 min). The supernatant was transferred into a new tube and diluted with an equal volume of isopropanol, incubated, mixed by gentle inversion, and incubated for an hour in the freezer. Samples were then centrifuged at 10,000 × g for two minutes. The settled DNA pellet was mixed with 300 µL of 70% ethanol and centrifuged at 10,000 × g for one minute. The supernatant was discarded, DNA air–dried, and the pellet was resuspended with 50 µL of TE buffer (1 M Tris buffer, pH 8.0, 0.5 M EDTA). DNA quality was checked by electrophoresis in 1% agarose gel in 0.5 × TAE buffer.

PCR Amplification and DNA Sequencing

Primer pairs ITS5 and ITS4 (White et al. 1990), Bt2a and Bt2b (Glass & Donaldson 1995), and cmd5 and cmd6 (Hong et al. 2006) were used to amplify ribosomal ITS regions, ß–tubulin (*BenA*) and calmodulin (*CaM*) genes, respectively (Table 1). The 25 µL PCR mix contained 1 × PCR buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP mix (Invitrogen), 0.2 µM of each primer, 1 U of *Taq* polymerase (Invitrogen), 1 µL DNA template and DEPC–treated water (Invitrogen). The PCR amplification was carried out in MyCycler™ Thermal Cycler System (Bio–Rad Laboratories). Amplification with the primer pairs ITS5 and ITS4, and cmd5 and cmd6 were performed using an initial denaturation step of 94 °C for 5 min, followed by 24 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension of 72 °C for 1 min. A final extension step at 72 °C for 7 min was then performed. Amplification with primer pairs Bt2a and Bt2b included denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 57.5 °C for 1 min, and extension at 72 °C for 1 min. A final extension step at 72 °C for 7 min was then used.

The amplified bands were subjected to gel electrophoresis with 1.5% agarose (Vivantis) dissolved in 0.5 × TAE buffer containing 2 µL GelRed solution (Biotium), run at 100 V for 30 min. The gel bands were visualized using the Molecular Imager GelDoc™ XR+ with Image Lab software (Bio–Rad Laboratories). PCR products were sent to 1st BASE (Malaysia) for DNA sequencing. A consensus sequence of the forward and reverse DNA sequences of the genes were made using Geneious software. Following this, a sequence similarity check was performed using the NCBI BLASTn software. Combined datasets of the three gene sequences were used to generate a phylogenetic tree through MEGA X software. Bootstrap analysis using 1,000 replications was used to assess the stability of the branches.

Detection of aflatoxin biosynthesis genes by PCR

Detection of *nor–1* and *aflR* gene regions involved in the pathway of aflatoxin biosynthesis was performed by PCR assay using gene–specific primers (Table 1). Cycling conditions for both primer pairs include initial denaturation of 94 ºC for 5 min, 40 cycles of denaturation at 94 ºC for 45 s, annealing at 60 ºC for 1 min, an extension of 72 ºC for 1 min, and a final extension of 72 ºC for 5 min. PCR products were visualized following the same procedure described earlier.

Table 1 List of primers used in this study.

Target sequences	Primer	5' to 3' sequence	Amplicon size (bp)	References
ITS Fungi	ITS5	GGAAGTAAAAGTCGTAACAAGG	~600	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC		
ß–tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	~550	Glass & Donaldson (1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		
Calmodulin	cmd5	CCGAGTACAAGGAGGCCTTC	~580	Hong et al. (2006)
	cmd6	CCGATAGAGGTCATAACGTGG		
aflR	AflRFor	CAACTCGGCGACCATCAGAG	~500	
	AflRRev	GGGAAGAGGTGGGTCAGTGT		
nor–1	nor1For	GGGATAGACGCCTGAGGAG	~200	
	nor1Rev	CTTCAGCGACGGTTAGTGCC		

Detection of aflatoxin by ELISA

Aflatoxin detection was performed using an ELISA–based method (Veratox, Neogen) following the manufacturer's protocol. Results were read in an ELISA reader using a 650 nm filter within 20 min upon the addition of the red stop solution. The controls form the standard curve's optical densities. The sample optical densities were plotted against the curve to calculate the exact concentration of aflatoxin.

Results

Fungal incidence in corn seed and milled products

Corn seeds and corn milled products such as corn cracks, grits, pericarp, darak and binlid were contaminated with an unknown *Aspergillus* sp. coded with the following: MC2–11, ME2–11, ME2–2–12 and ME2–2–11. The fungus was primarily isolated from surface–sterilized samples (internal portion of the samples). The highest incidence was in the pericarp (13%) and very minimal in other samples. It was observed only in crack samples (3%) of IPBVar6 Magdalena in non–surface sterilized samples (Fig. 1).

Fungus morphology and colony characteristics

At seven dpi, radial growth of the unknown *Aspergillus* sp. ranged from 37 mm–42 mm. The colonies were dark brown to brown with white mycelia at the margins in PDA. In MEA, the cultures were dark brown. They appeared to have a white mycelium that grew inconspicuously on the colonies giving them a grayish appearance. Reverse colonies on both media were brownish to light yellow. Radial growth at seven dpi ranged from 19–20 mm (Fig. 2A). The culture produced uniseriate phialides, globose conidial heads, smooth–walled stipes, and globose to subglobose vesicles. The stipes average width measured approximately 10.1 µm, and the vesicle diameter was

approximately 45–65 µm (av. 56 µm). The conidia were subglobose to globose with rough walls (echinulate) and measured 2.4–3.8 µm (av. 3.06 ± 0.4 µm) (Fig. 2B–C) (Noonim et al. 2008).

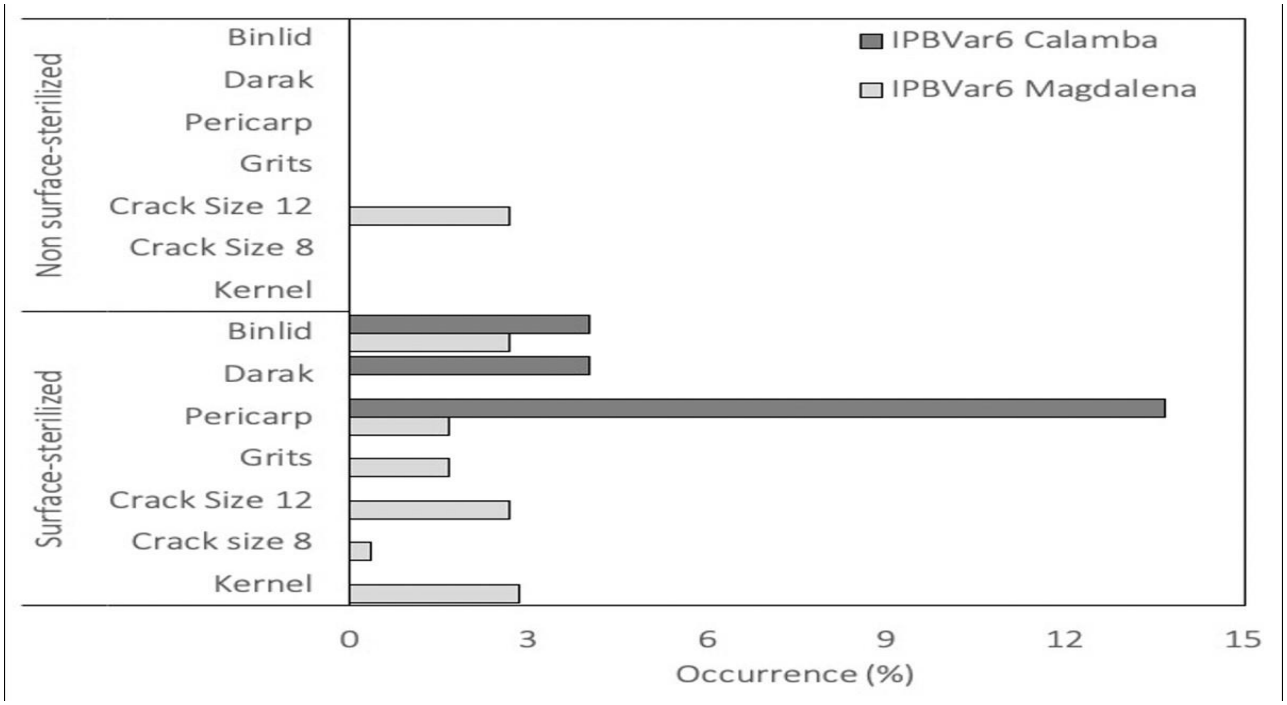

Fig. 1 – Occurrence (%) of the unknown *Aspergillus* sp. in surface–sterilized and non–surface sterilized corn–milled products of IPBVar6 Calamba and Magdalena.

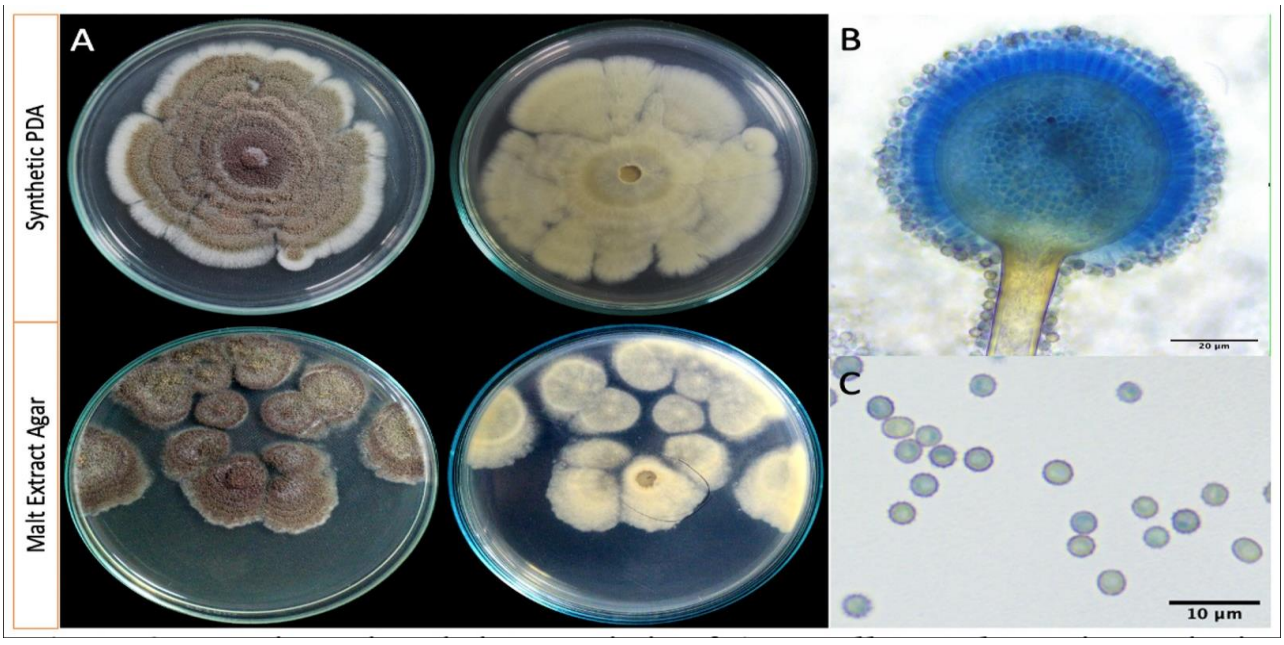

Fig. 2 – Morpho–cultural characteristic of *Aspergillus aculeatinus* in synthetic PDA and malt extract agar; obverse and reverse sides (A). Vesicle (B) and spores (C) were taken at 1000 × magnification.

DNA Sequences and Phylogenetic Analysis

A single fragment of approximately 600, 550, and 580 bp was amplified using the primers for ITS region, *BenA*, and *CaM* gene, respectively. Initial analysis of DNA sequences using the

BLASTN showed a 100% similarity on the ITS region to more than ten *Aspergillus* spp., such as *Aspergillus aculeatinus*, *A. aculeatus*, *A. assiutensis*, and *A. japonicus*. For the *BenA* and *CaM* regions, the sequences have 100% similarity to *A. aculeatinus.* A phylogenetic tree was constructed, using three concatenated gene sequences, which further delineated the species as *A. aculeatinus* (Fig. 3).

Presence of aflatoxin biosynthesis genes

The aflatoxin biosynthesis genes *nor–1* and *aflR*, which are both involved in the pathway of aflatoxin biosynthesis, were detected (Fig. 4) in all *A. aculeatinus* isolates used in the assay. On the other hand, aflatoxin was not detected in seven–day–old pure cultures of representative *A. aculeatinus* isolates using ELISA (Table 2).

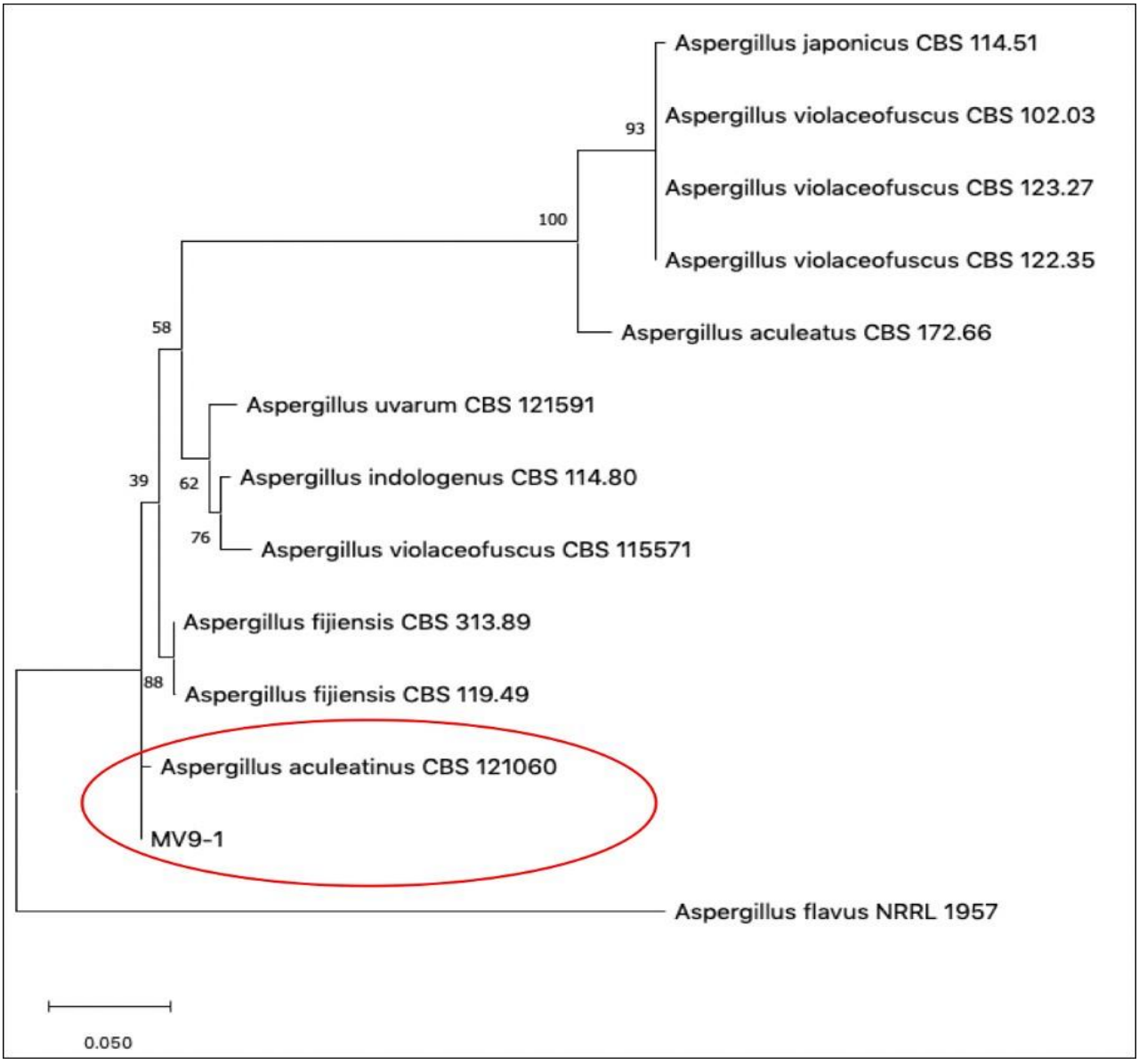

Fig. 3 – Phylogenetic tree generated by the maximum likelihood analysis with 1,000 bootstrap replicates (MEGA X) of *A. aculeatinus* isolate. Authentic sequences of *A. aculeatus* clade under *Aspergillus* section *nigri* described by Varga et al. (2011) retrieved from GenBank using concatenated partial sequences of ITS, *BenA*, and *CaM* gene regions. *Aspergillus flavus* was served as an outgroup.

Table 2 Aflatoxin determination in representative *Aspergillus aculeatinus* isolates by ELISA (Veratox, Neogen).

Isolate/Sample	Concentration (ppb)
A. aculeatinus MC2–11	0
A. aculeatinus ME2–11	0
A. aculeatinus ME2–2–12	0
A. aculeatinus ME2–2–11	0
A. flavus V6MD8–10 (control check)	>200

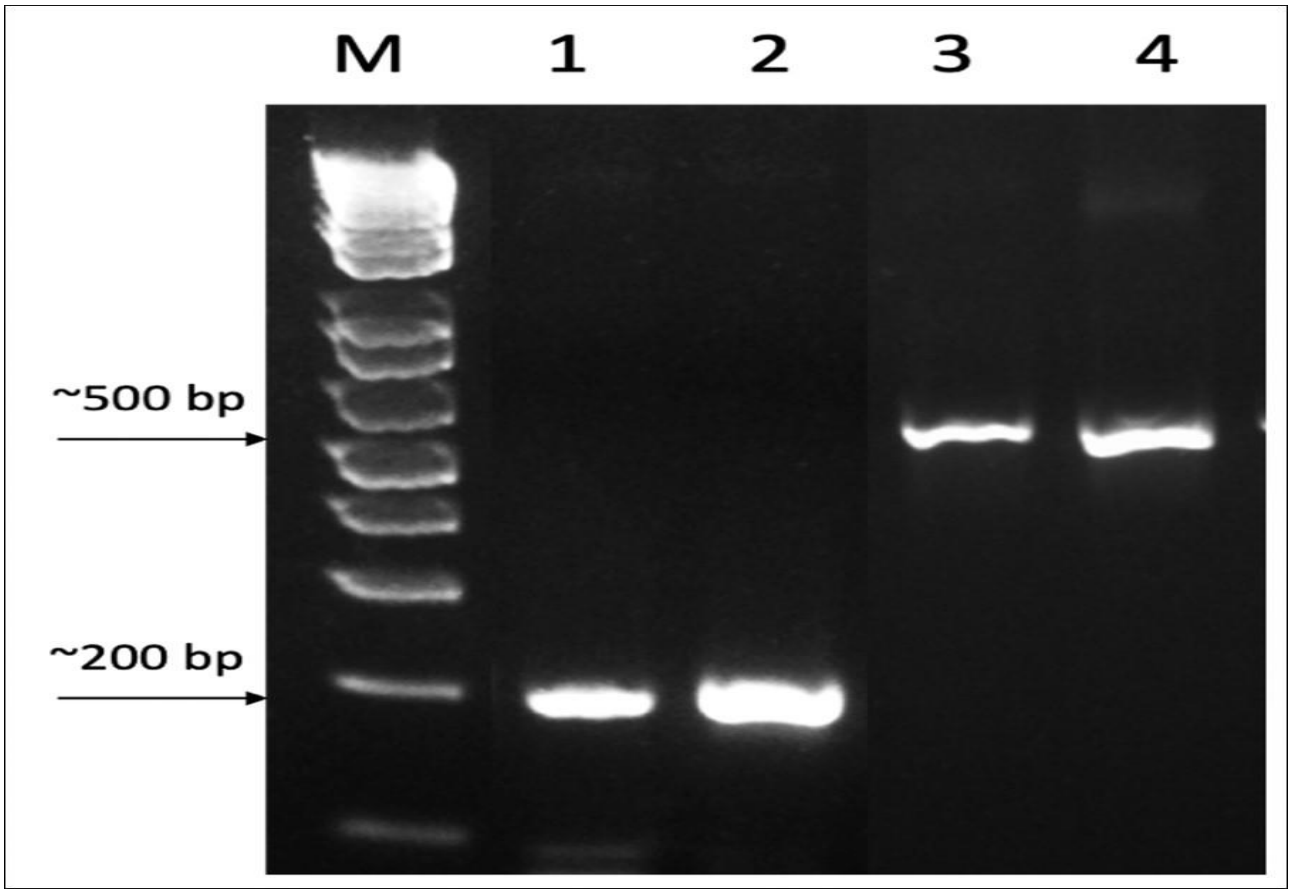

Fig. 4 – PCR analysis of representative *Aspergillus* isolates using *nor–1* (Lanes 1 and 2) and *aflR* (Lanes 3 and 4) specific primers with a product size of ~200 bp and ~500 bp, respectively.

Discussion

The morpho–cultural characteristics of *A. aculeatinus* reported in this study were in agreement with Noonim et al. (2008) descriptions, where conidia size was found to be the most distinguishable characteristic from other species ranging from 2–5 µm. Although morphological identification can be used for species differentiation, molecular identification aids in more accurate species identity, particularly in cryptic species like *Aspergillus* sp. (Alastruey–Izquierdo et al. 2012). Some *Aspergillus* species could be misidentified using macroscopic and microscopic characteristics (Zulkifli & Zakaria 2017). Noonim et al. (2008) reported the difficulty in differentiating the novel species from other uniseriate species such as *A. aculeatus* and *A. japonicus* using morphological or physiological characteristics because they showed the same growth rate in different media. In this study, the fungus was identified using combined DNA sequences of the ITS, *BenA* and CaM gene regions. The DNA sequences had high similarity (100%) with both *A. aculeatinus* and *A. aculeatus.* Using the authentic sequences of *Aspergillus* section *nigri* (Varga

et al. 2011), the phylogenetic tree constructed from the concatenated sequences of the three genes delineated the species identity as *A. aculeatinus*.

Aspergillus aculeatinus was reported as a novel species isolated from Arabica coffee beans from the North and Robusta coffee beans from the South of Thailand and garden soil from Japan (Noonim et al. 2008). In addition, *A. aculeatinus* was also found in complementary food samples based on maize and peanut in Nigeria (Ojuri et al. 2018), and in dried fruits such as apricot, plum, zibib from the Duhok governorate in Iraq (Saadullah & Abdullah 2014). In the Philippines, the *Aspergillus* species isolated from different crops were *A. flavus, A. parasiticus, A. carbonius, A. japonicus, A. ochraceous, A. niger, A. westerdijkiae, A. tamarii,* and *A. nomiu*s, where aflatoxins are produced mainly by *A. flavus* (Waing et al. 2015, Balendres et al. 2019; Hussien 2019). Hence, to our knowledge, this was the first report of *A. aculeatinus* in the country.

The consumption of contaminated corn could be detrimental to the consumer's health. In the Philippines, the warm temperature and high relative humidity are known to encourage mycotoxigenic fungi growth and mycotoxin production (Sales & Yoshizawa 2005). *Aspergillus aculeatinus* isolates in this study were found to contain the aflatoxin biosynthesis genes, *nor–1* and *aflR,* through PCR assay. However, in the ELISA assay, no aflatoxin was produced by these representative isolates. These results suggest that the presence of biosynthesis genes does not always indicate the presence of the toxin. There could be also several factors involved in the lack of toxin production in these isolates including temperature and media requirement of *A. aculeatinus*. Aflatoxin synthesis depends on a lot of factors that can modulate the gene expression responsible for coding enzymes controlling the biosynthesis pathway (Caceres et al. 2020). Environmental factors affect the gene expression involved in toxin production which activates different cell signaling pathways (Vlajkov et al. 2021).

In conclusion, the fungus *Aspergillus* is a cosmopolitan spore–forming microorganism that can produce some of the world's most important mycotoxins. In corn, *A. flavus* and *A. parasiticus* are the two commonly detected fungal contaminants. In this study, using a combined morpho–cultural and molecular characterization, *A. aculeatinus* was identified as a new fungal species contaminating corn seeds and milled products in the Philippines. While some aflatoxin biosynthesis genes were detected in the fungus, no aflatoxin was produced and detected. The identification of fungal contaminants is paramount to the successful mitigation of problems associated with potential mycotoxigenic fungi. This research indicates that other than the commonly detected *Aspergillus* species, *A. aculeatinus* also contaminates corn seeds and milled corn products.

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