Molecular Characterization of *Tamarindus indica* Associated with Fungal Diversity and Detection of Aflatoxin Biosynthesiser Genes

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HIGHLIGHTS

- Tamarind's acidity and low moisture enhanced its shelf stability.
- *Aspergillus aflatoxiformans* carried genes for aflatoxin biosynthesis.
- RFLP grouped the fungi but lacked species-level resolution.

ABSTRACT

Background: The tamarind tree (*Tamarindus indica*), valued for its nutritional and economic importance, faces significant food safety challenges due to fungal contamination and mycotoxin production. This study aims to characterize the toxigenic fungal flora of tamarind pulp to provide crucial information for developing improved management practices to ensure food safety and protect public health in the region.

Methods: Tamarind samples (n=180) collected from different locations were analyzed for pH, Titratable Acidity, and Moisture Content. Fungal diversity was assessed with emphasis on the molecular identification of *Aspergillus* species. The ammonia vapor test was used to detect aflatoxigenic isolates, while the presence of key aflatoxin biosynthesis genes (*aflD, aflM, aflO, aflP, aflQ,* and *aflR*) was investigated by Polymerase Chain Reaction amplification. Restriction Fragment Length Polymorphism analysis was applied to group isolates. The data were analyzed using the Kruskal-Wallis test in R software (version 4.4.3; R Foundation for Statistical Computing) to assess differences between groups. Statistical significance was determined at $p<0.05$.

Results: Tamarind was highly acidic (pH<3) with low Titratable Acidity (4-5%) and low water content (14-20%), which improves storage stability. Fungal analysis identified five species, namely *Monascus pilosus, Aspergillus melleus, Aspergillus aflatoxiformans, Aspergillus niger,* and *Aspergillus costaricensis* (the most frequently isolated species). Molecular analysis revealed amplification of four distinct fungal profiles, highlighting the usefulness of Restriction Fragment Length Polymorphisms for grouping isolates. However, closely related species such as *A. niger* and *A. costaricensis* were difficult to distinguish. Amplification of the *aflD, aflM, aflO, aflP, aflQ,* and *aflR* genes in *A. aflatoxiformans* isolates confirmed their ability to synthesise aflatoxins.

Conclusion: This study highlighted the vulnerability of tamarind to contamination by aflatoxin-producing fungi. Monitoring mycotoxin levels and improving storage practices are essential to ensure tamarind safety and protect consumer health.

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Introduction

The tamarind tree *Tamarindus indica*, a monotypic species in the *Fabaceae* family, is native to tropical Africa. Its widespread cultivation in tropical regions (Diallo et al., 2010) highlights its importance across various sectors, including nutrition, traditional medicine, and cultural practices. In West Africa, tamarind plays a crucial role not only in traditional diets and therapeutic but also in the economic sustainability of local communities (Hamacek et al., 2013; Samarou et al., 2022a,b; Stege et al., 2011). Tamarind pulp is rich in macronutrients and micronutrients, particularly essential amino acids, and represents an excellent source of vitamin C. It is also rich in pectin and organic acids, particularly tartaric acid, and is extensively used in various food preparations such as sauces, jams, and fruit pastes (Bakayoko et al., 2024b; Devi and Boruah, 2020; Grollier et al., 1998; Hamacek et al., 2013; Samarou et al., 2022b). These culinary applications underscore not only the nutritional richness of tamarind pulp but also its versatility in local food traditions.

Despite its benefits, tamarind fruit faces significant challenges primarily due to its susceptibility to microbial contamination. Mold, prevalent in tropical environments, poses a substantial threat during the harvesting, storage, and processing stages of tamarind. This contamination not only affects the quality of the fruit but also poses serious food safety risks due to the potential production of mycotoxins. These secondary toxic metabolites produced by certain molds can lead to severe health issues including immune disorders, liver damage, and cancer (Kabak, 2009; Kouadio et al., 2014; Mafe and Büsselberg, 2024). Therefore, understanding the microbial flora associated with tamarind fruit, especially molds capable of producing mycotoxins, is crucial for ensuring food safety.

In Côte d'Ivoire, tamarind pulp, often more or less dried, is used to make a traditional beverage known as "tomi." This beverage is typically prepared with water, pulp, and sugar, and is consumed without prior treatment, making it a cost-effective, accessible, and ready-to-drink option (Konan et al., 2022; Ouattara et al., 2016; Yao et al., 2021). However, the consumption of this artisanal beverage could pose health risks if certain molds present in tamarind pulp are mycotoxin producers. To date, there have been very few studies focusing on the identification of molds in tamarind fruit, and none specifically address mycotoxin-producing fungi.

To address this gap, it is essential to identify the specific fungal species present in tamarind pulp and investigate the potential presence of mycotoxin-producing species. This will help to develop effective quality and food safety management strategies. Although sequencing is one of the most successful methods for identifying fungal species, it

is prohibitively expensive when applied to large numbers of isolates. As a result, Restriction Fragment Length Polymorphisms (RFLP) analysis of the 5.8S region of the Internal Transcribed Spacer (ITS) region has emerged as the best alternative for grouping isolates, thereby reducing the number of strains requiring sequencing. In addition, this method has proven effective in identifying many fungal species and has been successfully extended to differentiate species within the same genus. Despite their low cost and relative ease of use, ITS-RFLP databases remain insufficient (Chanprasartsuk et al., 2013; Diguta et al., 2011, 2019).

This study aimed to characterize the toxigenic fungal flora of tamarind pulp to provide crucial information for developing improved management practices to ensure food safety and protect public health in the region.

Materials and methods

Sample collection

Tamarind samples were collected from wholesale markets in Abidjan, specifically from the Adjamé, Koumassi, and Abobo municipalities. From each of the three sampling sites, 60 tamarind samples (500 g each) were collected per wholesaler, resulting in a total of 180 samples. The samples were stored in coolers with ice packs at approximately 4 °C and transported to the laboratory for analysis.

pH measurement analysis

pH measurement was performed using the method described by Gacem (2011). A 20 g tamarind sample mixed with 40 ml distilled water was used to determine pH in 10 ml aliquots. Titratable Acidity (TA) was assessed according to ISO (1998) by titrating a 10 ml tamarind-water mixture with 1N sodium hydroxide (NaOH; S5881–500G, Sigma-Aldrich, USA) after adding phenolphthalein (P9750–25G, Sigma-Aldrich, USA). Moisture Content (MC) was determined by drying five g of tamarind at 105 °C for 24 h, following the method of Gacem (2011). Each experiment was carried out in triplicate.

Isolation of fungal and identification by conventional methods

Tamarind samples were inoculated by placing a seed directly onto Dichloran Rose Bengale Chloramphénicol (DRBC) agar (CM0727, Oxoid, UK) plates using sterile tweezers. Three Petri dishes per sample were prepared. The plates were incubated at 30 °C for 6 days, following the method described by Ghiasian et al. (2004). After incubation, characteristic mold colonies were purified by transferring a mycelium fragment from the colonies to the

center of a new DRBC agar (CM0727, Oxoid, UK) plate. This plate was then incubated at 30 °C for an additional six days. Approximately 470 isolates were examined through macroscopic and microscopic observations, and they were stored at -20 °C.

DNA extraction and Polymerase Chain Reaction (PCR) reaction

For PCR assays, DNA extraction from 6-week-old mycelium on DRBC agar (CM0727, Oxoid, UK) was performed using the Dneasy PowerSoil® DNA isolation kit (12888-100, Qiagen, Germany), according to the manufacturer's instructions. The PCR procedure targeting the 5.8S region of ITS region was conducted using the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTGATATGC). Each 50 µl PCR reaction contained 25 µl of one Taq 2X Master Mix (M0482S, New England Biolabs, UK), 0.5 µm of each primer, and 10-30 ng of fungal DNA. Amplification was carried out on a MasterCycler X50 (Eppendorf, Germany) with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 40 s. A final extension was performed at 72 °C for 5 min (Rao et al., 2020). The resulting PCR product was analyzed on a 1% agarose (A9539-100G, Sigma-Aldrich, USA) gel.

Taxonomic assignment of fungal isolates

PCR-amplified 5.8-ITS DNA fragments were sequenced using the primers ITS1 and ITS4. Eurofins Genomics (Germany) performed the sequencing of these fragments in both directions. The resulting nucleotide sequences have been submitted to the GenBank database, with accession numbers provided in Table 3. To identify the taxon, the sequences were compared with data from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1990).

DNA digestion

PCR products were digested with endonucleases *HaeIII* (R6175, Promega, USA), as well as *EcoRI* (R0101S, New England Biolabs, UK), *bslI* (R0555S, New England Biolabs, UK), and *Sau3AI* (R0169S, New England Biolabs, UK) for 4 h at 37 °C. These enzymes were selected based on their ability to differentiate between isolates, as determined from the analysis of ITS sequences in the GenBank database. The resulting restriction fragments were analyzed using 2% agarose (A9539-100G, Sigma-Aldrich, USA) gels, and DNA bands were visualized under UV light. The sizes of the fragments were estimated by comparison with the Quick-Load Purple 50 bp DNA ladder (N0556S, New England Biolabs, UK).

Qualitative screening for toxin production in Aspergillus

-Ammonia vapor method

The *Aspergillus* isolates were grown on Potato Dextrose Agar (PDA) (CM0139, Oxoid, UK) and incubated at 28±2 °C for a period of 7 days, with two replicates for each sample. Following the incubation, the petri dishes were inverted, and 2 ml of concentrated ammonia solution (105422-1L, Sigma-Aldrich, USA) was added to the lid of each inverted plate. The plates were then exposed to ammonia vapour for 10-15 min. After this exposure, any changes in colony colour were observed and documented (Shekhar et al., 2017).

Aflatoxin gene screening

The genes *aflD* (Nor1-F: ACGGATCACTTAGCCAGCAC, Nor1-R: CTACCAGGGGAGTT GAGATCC), *aflM* (Ver1-F: CCGTTTAGATGGCAAAGTGG, Ver1-R: CTTTCAGGTGACC GAACGAT), *aflO* (OmtB(F)-F: GCCTTGACATGGAAACCATC, OmtB(F)-R: CCAAGATG GCCTGCTCTTTA), *aflP* (Omt1-F: GCCTTGCAAACACACTTTCA, Omt1-R: AGTTGTTG AACGCCCCAGT), *aflQ* (Ord1(P)-F: CGACTGTTGGCCTTTTCATT, Ord1(P)-R: ATAGCG AGGTTCCAGCGTAA), and *aflR* (AflR-F: CGAGTTGTGCCAGTTCAAAA, AflR-R: AAT CCTCGCCCACCATACTA) (Abd El-Aziz et al., 2021; Lin et al., 2013) were targeted for amplification in *Aspergillus* isolates identified as positive using the Ammonia vapor method. The PCR conditions followed the protocol established by Rao et al. (2020), consisting of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 60 s at 55 °C, and 90 s at 72 °C, and concluded with a final extension at 72 °C for 7 min.

Data analysis

The data were analyzed using the Kruskal-Wallis test in R software (version 4.4.3; R Foundation for Statistical Computing) to assess differences between groups. Mean differences were evaluated, and statistical significance was determined at a significance level of $p<0.05$.

Results and discussion

The analysis revealed significant variations in pH, TA, and MC among the three districts (Table 1), reflecting the influence of local environmental conditions and processing methods on tamarind quality. Adjamé had a moderately acidic pH (2.2), higher than Koumassi (1.71) but lower than Abobo (2.40). These low pH values (below 3) indicate the acidic nature of tamarind attributed to the presence of organic acids, mainly tartaric acid, which imparts its characteristic sour taste (Hamacek et al., 2013; Obulesu

and Bhattacharya, 2011; Soloviev et al., 2004). Our results are in agreement with those of other authors who also found that tamarind has an acidic pH (Asoiro et al., 2017; Devi and Boruah, 2020; Yao et al., 2021). The low pH can also be explained by the fact that, after picking, the tamarind pods undergo uncontrolled fermentation due to lactic acid bacteria. These produce organic acids that lower the pH of the tamarind (Bakayoko et al., 2024a, b). Koumassi had the highest TA (4.79%), followed by Adjamé (4.46%), and Abobo (4.03%). This range (4-5%) is typical of tamarind and highlights its acid profile, which can vary slightly depending on environmental and processing factors. In terms of MC, Abobo recorded the

highest value (20.87%), while Adjamé (16.83%) and Koumassi (16.92%) had similar, lower values. This low MC could be explained by the fact that tamarind is a dried fruit (Asoiro et al., 2017; Devi and Boruah, 2020; Konan et al., 2022; Soloviev et al., 2004). Indeed, in Côte d'Ivoire, before tamarind is sold, the pods are dried in the sun (Aka et al., 2025). The low MC levels (14-20%) observed in all the samples are beneficial for shelf life, as they reduce enzymatic activity and microbial spoilage. This observation is consistent with previous findings, which indicate that dried or low-moisture foods (containing less than 25% MC) are more shelf-stable due to reduced water activity (Erkmen and Bozoglu, 2016).

Table 1: Physicochemical properties of tamarind pulps

| Districts	pH	TA (%)	MC (%)
Adjamé	$2.2\pm0,07$ [b]	4.46 ± 0.02 [b]	16.83 ± 0.55 [a]
Koumassi	1.71 ± 0.05 [a]	4.79 ± 0.52 [c]	16.92 ± 0.53 [a]
Abobo	2.40 ± 0.06 [c]	4.03 ± 0.10 [a]	20.87 ± 0.54 [b]

In a column, mean± Standard Deviation (SD) values followed by different superscript letters are significantly different according to the Kruskal-Wallis test ($p<0.05$).
MC=Moisture Content; TA=Titratable Acidity

PCR amplification of the 5.8S region of the ITS using primers ITS1 and ITS4, produced a 600-bp band (Figure 1) for all 470 fungal isolates. Based on macroscopic and microscopic characteristics, as well as RFLP profiles, five groups were identified. Group I was characterized by fluffy

yellow colonies with a white margin and a colorless reverse. Microscopically, it exhibited septate, branched mycelia, and biseriate conidial heads with globose vesicles (Table 2).

Table 2: Macroscopic and microscopic appearances of tamarind fungal isolates

Group	Macroscopic appearance	Microscopic appearance
Group I	Fluffy colonies, yellow with white margin, colorless reverse	Septate, branched mycelia; biseriate, radiate conidial heads; globose vesicles
Group II	Powdery green colonies with white margin, pale pink reverse, medium forms	Colorless, septate, branched mycelia; biseriate conidial heads with spherical vesicles
Group III	Flat gray-yellow colonies with colorless reverse, small forms	Colorless, septate mycelia; brush-like ramified conidial heads
Group IV	Granular black colonies with white margin, beige reverse, large forms	Septate, branched mycelia; fast-growing thallus; globose, brown conidia; vesicles sometimes slightly flattened
Group V	Powdery brown colonies spreading on medium, beige reverse	Septate, branched mycelia; globose conidiophores
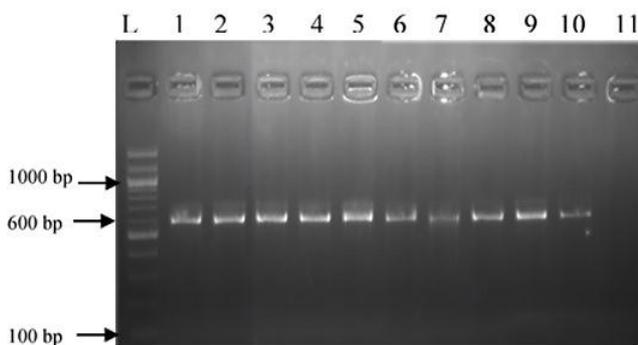
Figure 1: Polymerase Chain Reaction (PCR) product of the Internal Transcribed Spacer (ITS-5.8S) rDNA region. L: 100 bp DNA ladder; lanes 1-10: PCR products from 10 out of 470 fungal isolates; lane 11: water control. The gel shows a 600 bp PCR product corresponding to the ITS-5.8S rDNA region of the fungal isolates

Enzymatic digestion using *HaeIII, EcoRI, Sau3AI,* and *BslI* produced fragments of 350 and 80 bp, 310 and 280 bp, 320 and 250 bp, and 435 and 90 bp, respectively (Table 3). BLAST analysis of the 5.8S-ITS sequence identified the species as *Aspergillus melleus* (99.12-100% identity) from the subgenus *Circumdati* and section *Circumdati*, with a prevalence of 20%. The identification of *A. melleus* highlights its potential role as a tamarind contaminant. While no pathogenic potential is commonly attributed to this species, its adaptation to low-moisture environments may explain its presence in tamarind samples. Group II exhibited powdery green colonies with a white margin and pale pink reverse. Microscopically, it displayed colorless, septate, branched mycelium, and biseriate conidial heads with spherical vesicles. Enzymatic digestion revealed fragments of 260, 110, and 70 bp for *HaeIII*; 295 and 280 bp for *EcoRI*; 330 and 180 bp for *Sau3AI*; and 350 and 160 bp for *BslI*. This group was identified as *Monascus pilosus* (99.65-100% identity), a species from the genus *Monascus* within the family *Trichocomaceae*, with a prevalence of 15%. Although *M. pilosus* is not commonly associated with mycotoxin production, its presence highlights the diversity of fungal species capable of contaminating tamarind. Group III showed flat gray-yellow colonies with colorless reverse and small forms. Microscopically, it exhibited colorless, septate mycelium, and brush-like ramified conidial heads. Fragments of 290, 100, and 70 bp for *HaeIII*; 300 and 290 bp for *EcoRI*; 230, 150, and 90 bp for *Sau3AI*; and 380 and 180 bp for *BslI* were observed. BLAST analysis confirmed the species as *Aspergillus aflatoxiformans* (100% identity) (Table 3) from the section *Flavi*, with a prevalence of 5%. *A. aflatoxiformans* has been identified as a producer of aflatoxins B1, B2, G1, and G2 and is commonly detected in foods across Africa (Frisvad et al., 2019). Among the major mycotoxins, aflatoxins, classified as furanocoumarin-derived polyketides, are considered to be the most toxic and carcinogenic. Aflatoxins are commonly found contaminating various agricultural products and present significant health risks to both humans and livestock (Navale et al., 2021). Group IV showed granular black colonies with a white margin and beige reverse. It featured septate, branched mycelium, a fast-growing thallus, globose brown conidia, and slightly flattened vesicles. The restriction fragments obtained were 290, 90 and 70 bp for *HaeIII*; 300 and 290 bp for *EcoRI*; 230, 210 and 90 bp for *Sau3AI*; and 300, 180, and 70 bp for *BslI*. The species was identified as *Aspergillus niger* (99.62-100% identity) from the section *Nigri*, with a prevalence of 20%. *A. niger* is known for its ability to produce ochratoxin A (OTA), on natural substrates, although species within the *Nigri* section generally produce lower levels of this toxin. OTA is commonly formed during storage of crops and has been identified as neurotoxic, hepatotoxic, immunotoxic, genotoxic, embryotoxic, teratogenic, and carcinogenic in both humans and animals (Navale et al., 2021). Group V displayed powdery brown colonies spreading on the medium with a beige reverse. Microscopically, it featured septate, branched mycelium, and globose conidiophores. This group exhibited restriction patterns identical to Group IV. The species was identified as *Aspergillus costaricensis* (99.67–100% identity) from the section *Nigri*, with a prevalence of 40%.

A recent study by Bian et al. (2022) reclassified 14 *Aspergillus* species within the *Nigri* section into six species, with *A. costaricensis* being reclassified as *Aspergillus tubingensis*. The work of Kizis et al. (2014) showed that the polymorphism between *A. niger* and *A. tubingensis* could be distinguished using the enzyme *Rsa*I. Therefore, *A. niger* and *A. costaricensis* could potentially be distinguished in our study using the *Rsa*I enzyme. However, it should be noted that no previous study has documented the RFLP profiles of the fungi analyzed here. Therefore, this study contributes to the enrichment of existing RFLP profile databases for fungi and underscores the utility of combining morphological identification, RFLP profiling, and sequencing for fungal species characterization. While RFLP is cost-effective for reducing sequencing needs, its limitations in distinguishing closely related species, such as *A. niger* and *A. costaricensis*, were evident. The prominence of *Aspergillus* species across tamarind samples can be attributed to their ability to thrive in low-moisture environments, as observed in grains where moisture levels below 13% did not significantly inhibit fungal growth (Nji et al., 2022). The detection of mycotoxin-producing species, such as *A. niger* and *A. aflatoxiformans*, highlights potential health risks and underscores the importance of storage and quality control measures for tamarind products.

Table 3: Fungal identities and restriction profiles

Group	Restriction enzymes				Identity obtained after sequencing	% Identity	Prevalence (%)	GenBank accession number
	HaeIII	*EcoRI*	*Sau3AI*	*BslI*				
Group I	350+80	310+280	320+250	435+90	*Aspergillus melleus*	99.12-100	13.77	PQ512171
Group II	260+110+70	295+280	330+180	350+160	*Monascus pilosus*	99.65-100	11.33	PQ512170
Group III	290+100+70	300+290	230+150+90	380+180	*Aspergillus aflatoxiformans*	100	3.60	PQ512167
Group IV	290+90+70	300+290	230+210+90	300+180+70	*Aspergillus costaricensis*	99.67-100	45.65	PQ512168
Group V	290+90+70	300+290	230+210+90	300+180+70	*Aspergillus niger*	99.62-100	25.65	PQ512169

Cultures of *Aspergillus* from the section *Flavi* were exposed to ammonia vapor. The appearance of a plum-red coloration on the underside of Petri dishes after exposure to ammonium hydroxide is an indicator of aflatoxigenic potential. This color change was observed in *A. aflatoxiformans*. The color change is attributed to anthraquinone intermediates involved in aflatoxin biosynthesis, which react under alkaline conditions to produce a red hue. In contrast, non-aflatoxigenic isolates do not exhibit any color change, confirming their inability to produce aflatoxins (Ronoh et al., 2024). The observed variations in color intensity are likely linked to differences in toxin production levels among the isolates. The plum-red coloration observed in this study may indicate lower aflatoxin production levels. However, this qualitative method does not determine the specific type of aflatoxin produced by *A. aflatoxiformans*. The color of the cultures changed from beige to plum red following exposure (Figure 2).

Figure 2: Qualitative screening of aflatoxin production in *Aspergillus aflatoxiformans* plates exposed to ammonia vapor (Left: beige control, Right: color change to plum red in isolate)

To confirm the toxinogenic potential of these isolates, six key aflatoxin pathway genes were screened. The results revealed the presence of *aflD, aflM, aflO, aflP, aflQ,* and *aflR* gene (Figure 3) in our *A. aflatoxiformans* isolates. These genes are known to play a critical role in regulating aflatoxin biosynthesis in fungal species (Abd El-Aziz et al., 2021; Musangi et al., 2024). The amplification of these genes in the isolates confirms their potential to produce aflatoxins. These specific genes were selected as targets because of their fundamental involvement in the aflatoxin biosynthetic pathway. The *aflR* gene is an important regulator of the aflatoxin biosynthesis gene cluster. The presence of the *aflR* gene is still of concern because a presumably atoxigenic fungus may later acquire the ability to produce aflatoxins. In addition, aflatoxin production is known to be influenced by other factors such as pH, substrate, temperature, oxidative stress, and water availability. The *aflO* gene encodes an O-methyltransferase enzyme that converts demethylsterigmatocystin (DMST) to sterigmatocystin (ST) and dihydrodemethylsterigmatocystin (DHDMST) to dihydrosterigmatocystin (DHST) (Ronoh et al., 2024). Each gene in the aflatoxin biosynthetic pathway has a specific function: *aflD, aflF,* and *aflM* encode enzymes involved in early steps of toxin synthesis, while *aflO, aflP,* and *aflQ* are involved in intermediate and final conversions. The *aflR* gene acts as a key transcriptional regulator, coordinating the expression of the entire gene cluster (Abdel-Hadi et al., 2011).
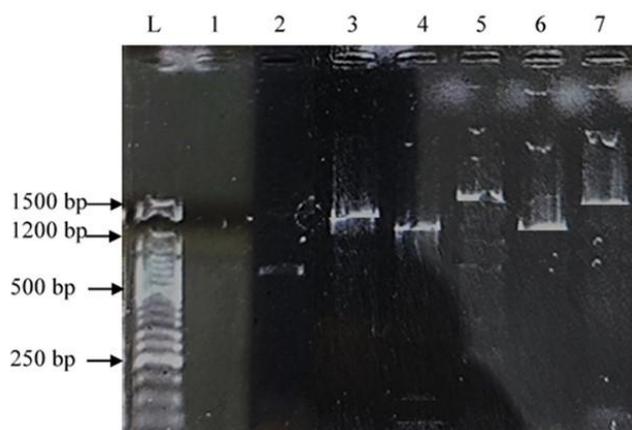
Figure 3: Polymerase Chain Reaction (PCR) product targeting the *aflR* gene in *Aspergillus aflatoxiformans*. L: 50 bp DNA Ladder; Lane 1: *aflD* gene; Lane 2: *aflQ* gene; Lane 3: *aflM* gene; Lane 4: *aflP* gene; Lane 5: *aflR* gene; Lane 6: *aflO* gene

Conclusions

The results of this study indicate that tamarind is a highly acidic fruit with a relatively low MC, making it well-suited for prolonged storage. The phenotypic identification of fungal colonies identified five species, namely *M. pilosus, A. melleus, A. aflatoxiformans, A. niger, and A. costaricensis*. Whereas, molecular identification yielded four distinct fungal profiles, highlighting that RFLP is helpful for grouping isolates. However, closely related species such as *A. niger and A. costaricensis* were difficult to distinguish. Contamination by *Aspergillus* species, particularly those capable of producing mycotoxins, raises food safety concerns, especially under suboptimal storage conditions. Future research should focus on monitoring mycotoxin levels in tamarind products and implementing better storage practices to reduce fungal contamination and ensure consumer safety.

Author contributions

B.B., T.L.S.A., and S.A.G. designed the study; B.B. acquired funding, conducted data curation and analyzed the data; B.B., T.L.S.A., K.M.C.N., M.K., and L.I.A.T. conducted the experimental work, and wrote the original draft; T.L.S.A. and S.A.G. supervised the research and validated the results; S.A.G. and M.K.D. reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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Ethical consideration

Not applicable.

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