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Molecular identification of aflatoxigenic *Aspergillus flavus* isolated from finished feed for farmed Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758)

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Abstract
Aflatoxins are toxic substances can contaminate food crops and pose a serious health threat to humans and livestock. Aflatoxinis are produced by *Aspergillus flavus* and *Aspergillus parasiticus* and these moulds can grow in improperly stored feed and feed with inferior quality of ingredients. Species identification of Aspergillus from the animal feed will give signs and types of mycotoxins produced. Therefore, ensuring adequate resources are available for testing and early diagnosis is very important for reducing serious health threat to humans and livestock. In this study, we isolated fungal isolates from five types finished feed for farmed Nile tilapia to identify fungal isolates to species level. Molecular method of assessment was done by using ITS1and ITS4 primers in the polymerase chain reaction (PCR) and sequences were analyzed by basic local alignment search tool analysis (BLAST). Our results indicate that all the five fungal isolates from Nile tilapia feed showed compatible (100%) homology with other *Aspergilus flavus* isolates on National Centre for Bioinformatics. Phylogenetic analysis was studied based on Neighbor-Joining and Maximum Likelihood methods with 1000 bootstrap values, using ITS region and the sequences showed that the *A. flavus* isolates from tilapia feed and other *A. flavus* isolates were clustered in the same clade.

Keywords: *Aspergillus flavus*, ITS, Nile tilapia feed, phylogenetic analysis

1. Introduction

Contamination of fish by fungi and their mycotoxins poses major health concerns to human and animals, particularly cereals used for livestock feed preparation (Ghani, 2013) [12]. Mycotoxins are fungal secondary metabolites reported to be potentially harmful to animals or humans. Livestock feed and feed ingredients contaminated with toxigenic fungi like *A. flavus* producing aflatoxins can be injurious for animals and humans health. Predominant Aspergillus sp. includes *A. flavus*, *A. parasiticus* and *A. nomius* species of fungi. Where *A. flavus,* produce aflatoxins B1 (AFB1), and B2 (AFB2), and *A. parasiticus,* which produce aflatoxins G1 (AFG1) and G2 (AFG2) toxins (Hesseltine, 1966) [18]. Animals exposed to aflatoxins through their diet undergo acute or chronic intoxication caused by mycotoxin ingestion (Chulze, 2010) [6]. Fish exposed to such mycotoxins or their producing fungi would have reduced growth rate, damaged liver, reduced immunity, and increased mortality. This may progress to a gradual decline in quality of reared fish stock and cause serious challenges to the aquaculture industry (Fallah *et al*., 2014) [11]. AFB1 might still be a serious concern in aquaculture since the vast use of plant sources in aquafeeds, and spread of AFB1 by lethal deposits in the fish may be a danger to humans as well (El-Sayed and Khalil, 2009; Manning, Li, and Robinson, 2005; Raghavan *et al*., 2011) [10, 23, 29]. However, there have been few studies reported on the contamination and the effects of AFB1 in farmed aquafeeds (Barbosa *et al*., 2013) [12]. The most common method used for the identification of Aspergillus isolates is based on the morphological characteristics is to sort the isolates into their respective groups or sections. However, these fungal species are much more similar to each other and accurate identification to species level could not be possible. Since accurate identification of the fungal pathogen is important, so a method is necessary for the identification and effective management of fungus related diseases in animals (Gherbawy and Voigt, 2010) [13].

Molecular based methods have used for differentiating the Aspergillus species, determining the phylogenetic analysis, characterization of isolates, identification of aflatoxigenic isolates from different food and feeds, diversity studies. (Almoammar *et al*., 2013; Chang *et al*., 2007; Baird *et al*., 2006; 2010) [1, 5, 3]. The most extensively used DNA target regions for discriminating Aspergillus species are the ones in the rDNA complex, mainly the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) (Samson *et al*., 2014) [31]. ITS region is the universal barcode of fungi and used for initial identification of Aspergillus, in fact, Aspergillus are among the best-studied fungi genetically (Dao *et al*., 2005) [7]. Because of its specificity and sensitivity, molecular methods such as PCR are the most suitable technique for fungus identification (Atkins and Clark, 2004) [2]. PCR method is described as more sensitive and specific methods for the detection of toxigenic moulds (Shapira *et al*., 1996; Haugland *et al*., 2002) [32, 17]. In the present study, we attempted isolation, PCR based identification, and species-level confirmation of aflatoxigenic *Aspergillus flavus* from Nile tilapia feed samples via sequencing of ITS region.

2. Materials and methods

2.1 Isolation and production of fungal mycelium

The fungal isolates were isolated from five tilapia feeds with the label TF1, TF2, TF3, TF4 and TF5 and subcultured in

Potato Dextrose Broth (PDB) for collecting mycelia of isolates. PDB was prepared by adding 6 g of PDB medium in 100 ml of distilled water. The suspension was mixed well, and heated with frequent agitation, boiled for one minute and sterilized at 121ºC (15 lbs. of pressure) (Mishra *et al*., 2014) [15]. Fungal strains were suspended in PDB and incubated in an orbital incubator at 28°C, 120 rpm for 24 to 48 hours or according to the growth of fungal mycelium, which was used immediately for DNA extraction.

2.2 Genomic DNA extraction

Genomic DNA was extracted from the fungal strains as per the salting-out method (Miller *et al*., 1998) [27]. Cultures were suspended in potato dextrose broth and incubated in an orbital incubator at 28°C, 120 rpm for 24 to 48 hours or according to the growth of fungal mycelium. Fungal mycelia were pelleted at 10,000 g for 10 minutes and then homogenized with 500 µl of Solution 1 (50 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 2% SDS) using a sterile toothpick. Then 5 µl Proteinase K (20 mg/ml) was added and the suspension was vortexed gently and incubated at 55°C in a water bath for 2 hours. After incubation, the suspension of the lysed cell in the eppendorf was chilled on ice for 10 minutes followed by addition of 250 µl of cold saturated NaCl (6 M) and gently mixed the lysate by slowly inverting the tube for several times. Again chilled on ice for 5 minutes and the concentrated DNA was separated by centrifugation at 8000 rpm for 15 minutes. The clear supernatant was transferred into a clean 1.5 ml eppendorf vial and added twice the volume of 100% AR grade ethanol. The mixture was incubated at 20°C for 12 to 24 hours to precipitate the DNA. The precipitated DNA was pelleted by centrifugation at 11,000 rpm for 15 minutes in a cooling centrifuge and washed the DNA pellet three times in 500 µl of cold 70% ethanol. DNA sample was dried under vacuum and dissolved in an appropriate volume of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and stored at -20 °C.

2.3 Estimation of quality and quantity of DNA

The quality and purity of DNA was evaluated by electrophoresis in 0.8% (w/v) agarose gel electrophoresis. DNA was quantified and qualified by measuring optical density (O.D) at 260 nm and 280 nm in UV Spectrophotometer (U-2900, Hitachi). The ratio of absorbance at 260 to 280 nm is an indication of DNA quality and the DNA will be having an absorbance 260/280 ≥ 1.8. When measured at 260 nm, 1 O.D of DNA = 50 µg/mL, hence the concentration of DNA can be calculated using the following formula.

Concentration of DNA (µg/mL) = Optical Density (O.D) of DNA at 260 nm * dilution factor * 50.

2.4 PCR amplification of ITS region

The Internal Transcribed Spacer (ITS) of rRNA gene was amplified using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTG ATATGC-3') (White *et al*., 1990). Details of the nucleotide sequences used for PCR amplification was given in Table 1. PCR amplifications were executed in 25 µl reaction volume containing 1X standard Taq buffer (10 mM Tris- HCl, 50 mM KCl, pH 8.3), 3.5 mM MgCl2, 0.2 mM dNTPs, 0.4 µM each primer, 1U Taq DNA polymerase (Fermentas, Inc.) and 1-2 µL of DNA template (10-100 ng). Reactions were performed on DNA Thermal cycler (Eppendorf, Germany). The PCR programme comprised of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C for 1 minute), annealing (56°C for 45 seconds) and extension (72°C for 1 minute) and a final extension (72°C for 10 minutes). Reaction mixtures devoid of DNA template were used as the negative control.

Table 1: Primer sequences used for amplification of ITS1 and ITS4 regions of rRNA genes of fungal isolates present in Nile tilapia feed samples.

Primer		Primer sequences	Molecular weight (bp)	Reference
ITS1	Forward	5'-TCCGTAGGTGAACCTGCGG-3'	560	White *et al*. (1990)
ITS4	Reverse	5'- TCCTCCGCTTATTG ATATGC-3'		

2.5 Agarose gel electrophoresis

The PCR products were electrophoresed on 0.8% agarose gel. Agarose gel was prepared in 1 X TBE (Tris base- 10.8 g, 0.5 M EDTA- 4 ml, Boric acid- 5.5 g, double distilled water- 100 mL, pH- 8.0) and fluorescent dye, ethidium bromide was added (20µg/ ml). After cooling to ear bearable temperature (45°C) the mixture was poured on to gel tray and allowed to solidify. Agarose gel was submerged in buffer tank filled with 1 X TBE buffer. Around 10 µl of PCR product was mixed well with 2 µl of 6 X gel loading dye (1% Bromophenol blue- 250 µl, glycerol- 300 µl, 1% xylene cyanol- 250 µl, double distilled water- 200 µl) and loaded into the wells. Electrophoresis was performed at a voltage of 3-5 volt/cm. The gel was visualized on a UV transilluminator using the Gel Doc XR system and the Quantity One programme (Bio-Rad).

2.6 DNA sequencing and sequence analysis

The ITS amplicons of the selected strains for sequencing were purified using Exo-SAP as per the manufacturer's instructions. Sequencing was carried out by employing the universal primers ITS1 and ITS4 with an ABI prism model 3700 Big Dye Sequencer (Applied Biosystems, USA) at SciGenom, Kochi, India. The nucleotide sequence obtained were assembled via Gene Tool Software and the sequences were compared with those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990) at the National Centre for Biotechnology Information (NCBI), USA and CBS- KNAW Fungal Biodiversity Centre, Academy of Arts and Sciences, Netherlands.

2.7 Phylogenetic analysis

To identify homologies, multiple sequence alignment was done using ClustalW alignment algorithm. Aligned sequences were arranged with MEGA X software (Kumar *et al*., 2018) [19] for phylogenetic analyses. The evolutionary history inferred using the Neighbor-Joining method (Saitou and Nei, 1987) [30] and the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) [19] and one thousand bootstrap replicates were performed.

3. Results

3.1 PCR analysis

In the present study, PCR amplification of the ITS region of rRNA genes with ITS1 and ITS4 primers yielded distinct DNA bands for all the isolates. DNA obtained was an intact band of good quality as shown in Fig 1. Amplification of the ITS regions from the five fungal isolates generated PCR products ranging in size from 564 to 568 bp (base pair). Among the 5 bands all isolates showing not much difference in number and size of fragments.

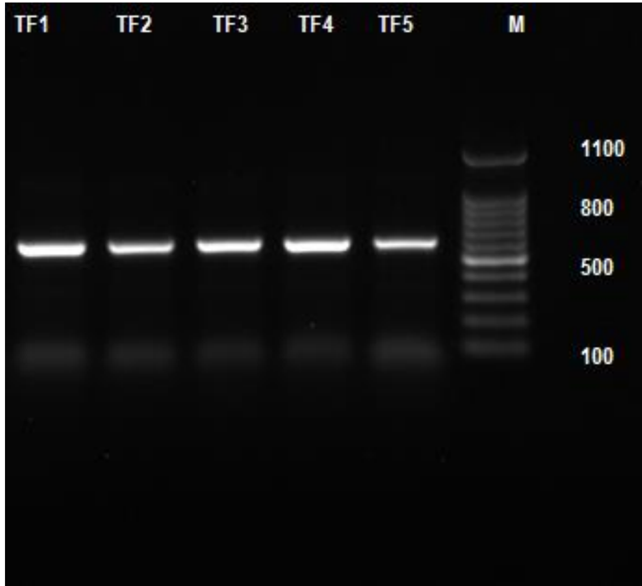

Fig 1: Representative 0.8% agarose gel electrophoresis of PCR amplification of ITS region of the fungal isolates from five test feeds. Key : lane 1- fungal isolates from TF1; lane 2 - TF2; lane 3- TF3; lane 4- TF4; lane 5- TF5; lane 6-1KB DNA marker.

3.2 Quantity estimation of extracted DNA

DNA quantity was evaluated by electrophoresis method by measuring optical density (O.D) at 260 nm and 280 nm in UV spectrophotometer. 260/280 ratio used to assess the Purity of DNA preparation (whether there is protein contamination or not) and DNA of five fungal isolates from five types of tilapia feed had an absorbance 260/280 ≥ 1.8.

3.3 Sequence analysis

PCR products of aflatoxigenic Aspergillus were randomly selected and sequenced at SciGenom. The QV score of nucleotides of all isolates is greater than 20, which means the probability that the base miscalled is not greater than 1%, is the acceptable standard for a good sequence reaction. Quality Value (QV) is a metric for determining quality sequencing data.

3.4 BLAST analysis

DNA sequences of each isolate were compared and analyzed their homology with standard nucleotide sequences deposited to NCBI gene bank using bioinformatics tool like NCBI, Basic Local Alignment Search Tool (BLAST) programme. The first fungal isolate (TF1) showed 100 % homology with *A. flavus* (MG799220.1) from NCBI at range of alignment 485-560, the second isolate (TF2) has 100% homology with *A. flavus* (MK397046.1) at range of alignment 480-544, the third isolate (TF3) has 100% homology with the *A. flavus* (MK299130.1) at range of alignment 480-600, the fourth isolate (TF4) showed 100% homology with *A. flavus* (MH244421.1) at range of alignment 506-533, and the fifth isolate (TF5) results in 100% homology with *A. flavus* (MK268123.1) at range of alignment, 484-619. Based on the maximum identity score sequence of fungal isolates from all tilapia feeds was 100% similar to the sequence of *A. flavus* available in NCBI database and sequences were aligned using multiple alignment software program Clustal W. The list of compatibility isolates, percent homology, accession number and name-identified organism given in Table 2. Based on sequence comparison, all the five ITS rRNA gene sequences of fungal isolates from tilapia feeds were confirmed as *Aspergillus flavus* and no other fungal species were found in the analysis.

Table 2: Compatibility of isolates of *Aspergillus flavus* with other isolates from NCBI

Isolate code	NCBI BLAST Hit results	Length of query sequences (bp)	Accession number	Maximum score	Total score	Query coverage	E value	Maximum Identity
TF1	*A. flavus* isolate	560	MG799220.1	896	896	100 %	0.0	100 %
TF2	*A. flavus* isolate	544	MK397046.1	887	887	100 %	0.0	100 %
TF3	*A. flavus* isolate	600	MK299130.1	887	887	100 %	0.0	100 %
TF4	*A. flavus* isolate	533	MH244421.1	935	935	100 %	0.0	100 %
TF5	*A. flavus* isolate	619	MK268123.1	894	894	100 %	0.0	100 %

3.5 Phylogenetic analysis

The distance matrix was generated and the phylogenetic tree constructed using Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar *et al.* 2018) [19]. The reference strains were *A. flavus* (MH244421.1), *A. flavus* (MK299130.1), *A. flavus* (MK397046.1), *A. parasiticus* (MK165724.1), *A. niger* (MK461093.1), *A. fumigatus* (MK461083.1), *A.clavatus* (MH170876.1), *A. arachidicola* (MH345960.1), *A.bombycis* (MH863014.1) and *A.tamarii* (MK332591.1). In the present case, isolates of *Alternaria alternata* (MK460949.1) was used as an outgroup to interpret the clustering of isolates as distinct or related out-group of the genus.

3.5.1 Evolutionary analysis by Maximum Likelihood Method

The evolutionary relationship between the DNA from the isolates sequenced depicted distinct clades. Sequence analysis also revealed inter and intra species diversity among *A. flavus* from same and different sources. Sequences yielded distinct clades revealing inter and intra species similarity and diversity among Aspergillus sp. Evolutionary history inferred using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993) [19]. Phylogenetic tree with the highest log likelihood (-2678.96) was shown. Matrix of pairwise distances was measured using the Maximum Composite

Likelihood (MCL) approach and choose the topology with superior log-likelihood value. In addition, the tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 16 nucleotide sequences (Fig. 2). Codon positions included were 1st+2nd+3rd+Noncoding. There were 653 positions in the final dataset.

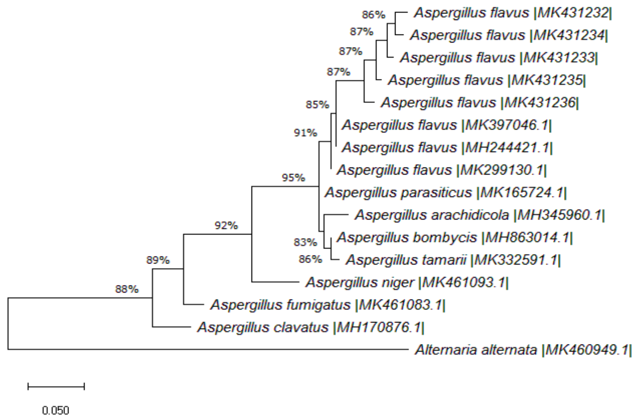

Fig 2: Phylogenetic tree based on ITS region of rRNA gene analysis using MCL method.

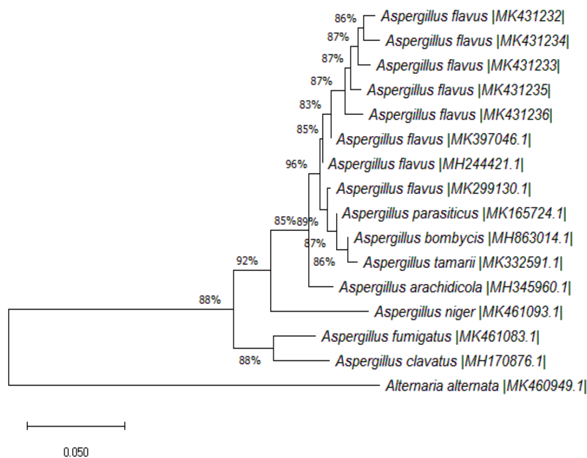

Fig 3: Phylogenetic tree based on ITS region of rRNA gene analysis using NJ method.

3.5.2 Evolutionary relationships of taxa- Neighbor-Joining method

Based on the NJ phylogenetic tree, the isolates of the same species were grouped in the same clade or sub-clade (Fig. 3) and the NJ tree formed clades and sub-clades. The evolutionary history was done by using the Neighbor-Joining method (Saitou and Nei, 1987) [30]. NJ phylogenetic tree showed an unrooted phylogenetic tree in cladogram format with branch lengths using a Neighbor-Joining method. The optimal tree with the sum of branch length was 0.56823677 was shown. The phylogenetic tree was drawn to scale, and branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. This analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions removed for each sequence pair (pairwise deletion option) and there was a total of 653 positions in the final dataset.

3.6 Nucleotide sequences accession numbers.

The ITS1–5.8S–ITS4 gene complex sequences of *A. flavus* isolates from tilapia feeds were submitted in to the National Center for Biotechnology Information GenBank. The assigned sequence accession numbers of *A. flavus* isolates were MK431232 (TF1), MK431233 (TF2), MK431234 (TF3), MK431235 (TF4) and MK431236 (TF5).

4. Discussion

The present study showed that *A. flavus* isolates were present in all feed used for tilapia culture. Presence of *A. flavus* species may be related to high temperature and high relative humidity as India is located in the tropics. Higher relative humidity, as well as conductive temperature, can considerably support the growth of Aspergillus (Shehu and Bello, 2011) [33]. Therefore, storage conditions of animal feed need to be under suitable environmental conditions to prevent the growth of Aspergillus as well as the growth of other storage fungi. Molecular methods have broadly applied in the identification of a large number of Aspergillus species. DNA amplification and DNA sequence analysis is a prevailing tool in taxonomy studies. In the present study, DNA was extracted from the culture and positive result amplification was obtained from mycelium of aflatoxigenic *A. flavus* isolated from the five types of Nile tilapia feed. PCR amplification of 5.8S rRNA gene done for characterization and identification of Aspergillus isolates firstly because of available universal fungal primers based on conserved regions of 5.8S rRNA. Secondly, the large numbers of 5.8S rRNA sequence are easily available in NCBI GenBank, which help to make similarity searches convenient.

In the present study, the compatibility of all analyzed isolates with the same standard type isolates of *A. flavus* could be related to the high similarity of ITS copy with the standard copy of *A. flavus* isolates. However, *A. flavus* species are difficult to differentiate even genetically. *A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae* have shown to possess high degrees of DNA similarity and similar genome size. Furthermore, *A. flavus* and *A. oryzae*, and *A. parasiticus* and *A. sojae,* considered virtually impossible to discriminate, since their DNA relatedness found to be of 100% and 91%, respectively (Kurtzman *et al.*, 1986; Kurtzman *et al.*, 1987) [20, 21]. This process considers as the rapid method for detection of aflatoxigenic *A. flavus* in selected fish feeds as compared with other methods. *A. flavus* detection by PCR is more accurate, sensitive, specific and less laborious (Shweta *et al.*, 2013) [34]. Several such studies by the use of PCR technology for the detection and diagnosis of fungi by using the internal transcribed spacer (ITS) have already been published (Marek *et al.*, 2003; Haughland *et al.*, 2004; Druzhinina *et al.*, 2005) [24, 8] which also support the present study. Godet and Munaut (2010) [14] also carried out similar studies recently in differentiating *A. flavus, A. parasiticus, A. tamari* and *A. nomius* by PCR-method. Likewise, Leema *et al.* (2010) [22] confirmed that molecular methods *i.e*, amplification of the internal transcribed spacer (ITS) regions for the identification of *A. flavus.* Aspergillus isolates further confirmed by molecular methods (Somashekar *et al.*, 2004; Midorikawa *et al.*, 2008) [35, 26]. Specific primers were used for identification of all the fungal isolates present in feeds. Primers ITS1 and ITS4

were designed from ribosomal RNA internal transcribed spacers (White *et al*., (1990). Suggestions made by Martinez-Culebras and Ramon (2007) [25], Varga *et al*. (2011) [38] and El Khoury *et al*. (2011) [9] on phylogenetic analysis using ITS and by using beta-tubulin genes can be adopted in developing a differential relationship between closely related species of fungi like *A. flavus* and *A. parasiticus* (Msiska, 2008) [28]. The result of the present study revealed that the sequence information and ITS region could use for authentic identification for molecular characterization of fungal isolates. The five fungal isolates from all tilapia feed showed high similarity with *A. flavus* based on nucleotide homology and phylogenetic analysis.

5. Conclusions

The molecular method employed for the genomic DNA isolation resulted in high-quality DNA from the fungal isolates. Universal eukaryotic primers (ITS1 and ITS4) used for the amplification of ITS regions and 5.8S rRNA was successfully amplified fungal genomic DNA. Sequence analysis was done and the sequence profile was obtained in FASTA format. Majority of the hits from the BLAST search were from a group of fungus belonging to *A. flavus*. The sequences were aligned with ClustalW and the relatedness between the sequences was determined. The evolutionary history was inferred using the Neighbor-Joining method and Maximum Likelihood method. The sequences were submitted in the NCBI gene bank and got accession numbers. Correct and rapid identification of Aspergillus from livestock feed is important particularly to detect and identify toxigenic species to employ correct treatment of contaminated feed and feed ingredients. Therefore, molecular method of identifications need to apply to avoid species misidentification.

6. Acknowledgment

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7. Conflict of Interest

We declare that we have no conflict of interest.

8. References

1. Almoammar H, Bahkali AH, Abd-Elsalam KA. A polyphasic method for the identification of aflatoxigenic' Aspergillus' species isolated from Camel feeds. Australian Journal of Crop Science. 2013; 7(11):1707.
2. Atkins SD, Clark IM. Fungal molecular diagnostics: a mini review. J Appl Genet. 2004; 45(1):3-15.
3. Baird RE, Trigiano RN, Windham G, Williams P, Kelley R, Abbas HK. *et al*. Comparison of aflatoxigenic and nonaflatoxigenic isolates of Aspergillus flavus using DNA amplification fingerprinting techniques. Mycopathologia. 2006; 161(2):93-99.
4. Barbosa TS, Pereyra CM, Soleiro CA, Dias EO, Oliveira AA, Keller KM. *et al*. Mycobiota and mycotoxins present in finished fish feeds from farms in the Rio de Janeiro State, Brazil. International Aquatic Research. 2013; 5(1):3.
5. Chang PK, Matsushima K, Takahashi T, Yu J, Abe K, Bhatnagar D. *et al*. Understanding nonaflatoxigenicity of Aspergillus sojae: a windfall of aflatoxin biosynthesis research. Applied microbiology and biotechnology. 2007; 76(5):977-984.
6. Chulze SN. Strategies to reduce mycotoxin levels in maize during storage: a review. Food Additives and Contaminants. 2010; 27(5):651-657.
7. Dao HP, Mathieu F, Lebrihi A. Two primer pairs to detect OTA producers by PCR method. International Journal of Food Microbiology. 2005; 104(1):61-67.
8. Druzhinina IS, Kopchinskiy AG, Komoń M, Bissett J, Szakacs G, Kubicek CP. *et al*. An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. Fungal Genetics and Biology. 2005; 42(10):813-828.
9. El Khoury A, Atoui A, Rizk T, Lteif R, Kallassy M, Lebrihi A. *et al*. Differentiation between Aspergillus flavus and Aspergillus parasiticus from Pure Culture and Aflatoxin- Contaminated Grapes Using PCR- RFLP Analysis of aflR- aflJ Intergenic Spacer. Journal of food science. 2011; 76(4):M247-M253.
10. El-Sayed YS, Khalil RH. Toxicity, biochemical effects and residue of aflatoxin B1 in marine water-reared sea bass (Dicentrarchus labrax L.). Food and chemical toxicology. 2009; 47(7):1606-1609.
11. Fallah AA, Pirali-Kheirabadi E, Rahnama M, Saei-Dehkordi SS, Pirali-Kheirabadi K. *et al*. Mycoflora, aflatoxigenic strains of Aspergillus section Flavi and aflatoxins in fish feed. Quality Assurance and Safety of Crops & Foods. 2014; 6(4):419-424.
12. Ghani AW. Grain and Feed Annual: Global Agricultural Information Network, GAIN Report. USDA Foreign Agricultural Service, 2013.
13. Gherbawy Y, Voigt K. eds., Molecular identification of fungi (pp. vii-xi). Berlin: Springer, 2010.
14. Godet M, Munaut F. Molecular strategy for identification in Aspergillus section Flavi. FEMS microbiology letters. 2010; 304(2):157-168.
15. Gontia-Mishra I, Tripathi N, Tiwari S. A simple and rapid DNA extraction protocol for filamentous fungi efficient for molecular studies, 2014.
16. Haugland RA, Brinkman N, Vesper SJ. Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. Journal of Microbiological Methods. 2002; 50(3):319-323.
17. Haugland RA, Varma M, Wymer LJ, Vesper SJ. Quantitative PCR analysis of selected Aspergillus, Penicillium and Paecilomyces species. Systematic and Applied Microbiology. 2004; 27(2):198-210.
18. Hesseltine CW, Shotwell OL, Ellis JJ, Stubblefield RD. Aflatoxin formation by Aspergillus flavus. Bacteriological reviews. 1966; 30(4):795.
19. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution. 2018; 35(6):1547-1549.
20. Kurtzman CP, Horn BW, Hesseltine CW. Aspergillus nomius, a new aflatoxin-producing species related to Aspergillus flavus and Aspergillus tamarii. Antonie van leeuwenhoek. 1987; 53(3):147-158.
21. Kurtzman CP, Smiley MJ, Robnett CJ, Wicklow DT. DNA relatedness among wild and domesticated species in the Aspergillus flavus group. Mycologia.

1986; 78(6):955-959.
22. Leema G, Kaliamurthy J, Geraldine P, Thomas PA. Keratitis due to Aspergillus flavus: Clinical profile, molecular identification of fungal strains and detection of aflatoxin production. Molecular vision, 2010, 16:843.
23. Manning BB, Li MH, Robinson EH. Aflatoxins from moldy corn cause no reductions in channel catfish Ictalurus punctatus performance. Journal of the World Aquaculture Society. 2005; 36(1):59-67.
24. Marek P, Annamalai T, Venkitanarayanan K. Detection of Penicillium expansum by polymerase chain reaction. International Journal of Food Microbiology. 2003; 89(2-3):139-144.
25. Martinez-Culebras PV, Ramon D. An ITS-RFLP method to identify black Aspergillus isolates responsible for OTA contamination in grapes and wine. International Journal of Food Microbiology. 2007; 113(2):147-153.
26. Midorikawa GEO, Pinheiro MRR, Vidigal BS, Arruda MC, Costa FF. *et al*. Characterization of Aspergillus flavus strains from Brazilian Brazil nuts and cashew by RAPD and ribosomal DNA analysis. Letters in applied microbiology. 2008; 47(1):12-18.
27. Miller SA, Dykes DD, Polesky HFRN. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic acids research. 1988; 16(3):1215.
28. Msiska Z. The β-tubulin gene as a means to discriminate speciesand verify evolutionary patterns of arbuscular mycorrhizal fungi. West Virginia University, 2008.
29. Raghavan PR, Zhu X, Lei W, Han D, Yang Y, Xie S. *et al*. Low levels of aflatoxin B1 could cause mortalities in juvenile hybrid sturgeon, Acipenser ruthenus ♂ × A. baeri. Aquaculture Nutrition. 2011; 17(2):39- 47.
30. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution. 1987; 4(4):406-425.
31. Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH. *et al*. Phylogeny, identification and nomenclature of the genus Aspergillus. Studies in mycology. 2014; 78:141-173.
32. Shapira R, Paster N, Eyal O, Menasherov M, Mett A. Salomo R. Detection of aflatoxigenic molds in grains by PCR. Appl. Environ. Microbiol. 1996; 62(9):3270-3273.
33. Shehu K, Bello MT. Effect of environmental factors on the growth of Aspergillus species associated with stored millet grains in Sokoto. Nigerian Journal of Basic and Applied Sciences, 2011, 19(2).
34. Shweta S, Madhavan S, Paranidharan V, Velazhahan R. Detection of Aspergillus flavus in maize kernels by conventional and real-time PCR assays. International Food Research Journal. 2013; 20(6):3329.
35. Somashekar D, Rati ER, Anand S, Chandrashekar A. Isolation, enumeration and PCR characterization of aflatoxigenic fungi from food and feed samples in India. Food microbiology. 2004; 21(6):809-813.
36. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular biology and evolution. 1993; 10(3):512-526.
37. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research. 1994; 22(22):4673-4680.
38. Varga J, Frisvad JC, Samson RA. Two new aflatoxin producing species, and an overview of Aspergillus section Flavi. Studies in Mycology. 2011; 69:57-80.
39. White TJ, Bruns T, Lee SJWT, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. PCR protocols: a guide to methods and applications, 1990, pp.315-22.