

Genetic Diversity of *Aspergillus* spp. in Stored Grains Using RAPD-PCR Technique

Saif Talib Jasim

Al-Qaim Education Dept., General Directorate of Education in Anbar, Ministry of Education, Iraq.

*e-mail: Dr.saifmyco@gmail.com

ABSTRACT

Background: *Aspergillus* species are the dominant storage fungi of cereal grains and include aflatoxigenic and opportunistic human pathogens. Data on the genetic structure of grain-borne *Aspergillus* populations in Iraq remain limited. **Objectives:** To isolate and identify *Aspergillus* spp. from stored wheat, maize and rice in Iraq and to assess their intra- and interspecific genetic diversity using random amplified polymorphic DNA (RAPD)-PCR. **Methods:** Ninety stored grain samples (30 each of wheat, maize and rice) were collected from warehouses and retail markets in Haditha, Hit, Ramadi and Rutba, Anbar Governorate, Iraq, between January and March 2026. Fungi were isolated by direct and dilution plating, identified morphologically and confirmed by ITS and calmodulin sequencing, and section *Flavi* isolates were screened culturally for aflatoxigenic potential. Genomic DNA of 36 isolates was amplified with five decamer RAPD primers. Bands were scored as binary data and analysed by polymorphism indices, polymorphic information content (PIC), Jaccard similarity, UPGMA clustering, principal coordinate analysis and analysis of molecular variance (AMOVA). **Results:** *Aspergillus* was recovered from 47 of 90 samples (52.2%). *A. flavus* was the dominant species (36.4% of 55 isolates), and 55.6% of section *Flavi* isolates were presumptively aflatoxigenic. The five primers generated 58 bands, of which 48 (82.8%) were polymorphic, with PIC values of 0.22–0.44. Jaccard similarity ranged from 0.31 to 0.91. UPGMA (cophenetic $r = 0.89$) resolved three clusters at 65% similarity that corresponded largely to species. AMOVA attributed 82% of the variation to differences within grain sources and 18% to differences among them ($\Phi_{PT} = 0.18$; $P = 0.018$). **Conclusion:** *Aspergillus* populations in stored Iraqi cereals are genetically diverse and structured mainly by species rather than by grain source. RAPD-PCR is a rapid, low-cost tool for baseline surveillance of storage *Aspergillus* and should be combined with aflatoxin-gene PCR for risk assessment.

Keywords: *Aspergillus*; Random Amplified Polymorphic DNA Technique; Genetic Variation; Edible Grain; Food Storage.

Article Information

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INTRODUCTION

Cereal grains are the main dietary staple in Iraq, and their quality depends heavily on post-harvest storage. Worldwide, a large share of food crops carries detectable mycotoxins, and levels above regulatory limits are frequent (1). Storage fungi, mainly *Aspergillus* and *Penicillium*, colonise grain when moisture and temperature are poorly controlled, causing loss of germination, discolouration, heating and toxin accumulation (2).

The genus *Aspergillus* contains the most important storage moulds. *A. flavus* and *A. parasiticus* produce aflatoxins, the most potent natural hepatocarcinogens, while *A. niger*, *A. ochraceus* and *A. carbonarius* produce ochratoxin A (3). *A. flavus* is also the second cause of invasive and allergic aspergillosis after *A. fumigatus*, and is a leading cause of fungal sinusitis and keratitis in hot, dry regions (4). Grain stores therefore act as reservoirs of

airborne conidia that link food contamination to human exposure, which makes them relevant to epidemiological surveillance (4). Natural antifungal agents against these species are an active research area (5).

Species identification in *Aspergillus* is difficult because sections such as *Flavi* and *Nigri* contain cryptic species that look alike. Current taxonomy relies on a polyphasic approach with calmodulin, β -tubulin and ITS sequences (6,7). Within species, isolates also differ in sclerotial type, toxigenicity and virulence, so morphology alone cannot describe population structure (7).

In neighbouring Egypt, *Aspergillus* section *Flavi* dominates the mycobiota of stored barley and maize, and many isolates carry aflatoxin biosynthesis genes (8). Aflatoxin B1 is also the mycotoxin of greatest concern in Egyptian maize (9). In Iraq, aflatoxigenic *A. flavus* and *A. niger* have been recovered from wheat stored in silos in Baghdad and Al-Muthanna (10,11), *A. flavus* was the most frequent *Aspergillus* species on stored maize around Baghdad (12), and aflatoxin B1 was detected in 60% of milled rice samples marketed in Duhok (13). However, most of these regional studies, including those from Iraq, report species frequency and toxin levels rather than genetic variation among isolates.

Random amplified polymorphic DNA (RAPD)-PCR uses single short primers of arbitrary sequence to amplify anonymous loci across the genome, without prior sequence knowledge (14,15). It is fast, cheap and needs little DNA, which suits laboratories in low-resource settings. RAPD has separated closely related *Aspergillus* taxa (16), produced species-specific patterns for *A. flavus*, *A. fumigatus* and *A. niger* (17,18), and revealed high genetic diversity in *A. flavus* from wheat, maize and barley in Saudi Arabia (19–21). Egyptian and Iraqi studies have applied RAPD to soil *Aspergillus* spp., *A. flavus* and *A. niger* (22–24). Sequence-based work on Kenyan maize

likewise showed that *A. flavus* populations from a single crop are genetically heterogeneous (25).

To our knowledge, no study has compared the RAPD-based genetic diversity of *Aspergillus* spp. across the three main stored cereals in Iraq within one sampling frame. This study therefore aimed to (i) determine the frequency of *Aspergillus* spp. in stored wheat, maize and rice; (ii) estimate intra- and interspecific genetic diversity of the isolates with RAPD-PCR; and (iii) test whether genetic variation is structured by species or by grain source.

METHODS

Study design and sampling

This cross-sectional study was conducted between January and March 2026. A total of 90 stored grain samples were collected: wheat (*Triticum aestivum*, $n = 30$), maize (*Zea mays*, $n = 30$) and rice (*Oryza sativa*, $n = 30$). Samples came from silos, governmental warehouses and retail markets in the districts of Haditha, Hit, Ramadi and Rutba, Anbar Governorate, Iraq. Each sample (about 1 kg) was a composite of five sub-samples drawn from the top, middle and bottom of the storage unit with a sterile probe. Samples were placed in sterile paper bags, labelled with source, storage period and date, kept at 4 °C and analysed within 72 h. Grain moisture content was measured by oven-drying 10 g at 105 °C to constant weight.

Isolation of fungi

Two complementary methods were used. For direct plating, 100 grains per sample were surface-disinfected in 1% sodium hypochlorite for 1 min, rinsed three times in sterile distilled water, blotted dry and placed on dichloran rose bengal chloramphenicol (DRBC) agar, 10 grains per plate (8). For dilution plating, 10 g of ground grain were homogenised in 90 mL of 0.1% peptone water, serially diluted to 10^{-4} , and 0.1 mL of each dilution was spread on DRBC in duplicate. Plates were incubated at 28 ± 2 °C in the dark for 5–7 days. Counts were expressed as

colony-forming units per gram (CFU/g). Isolation frequency (Fr) and relative density (RD) were calculated as follows:

$$\begin{aligned} Fr (\%) &= \frac{\text{Number of samples yielding the species}}{\text{Total number of samples}} \times 100 \\ RD (\%) &= \frac{\text{Number of isolates of the species}}{\text{Total number of fungal isolates}} \times 100 \end{aligned}$$

All *Aspergillus* colonies were purified by single-spore isolation and maintained on potato dextrose agar (PDA) slants at 4 °C and in 20% glycerol at -20 °C.

Morphological identification

Isolates were three-point inoculated on Czapek yeast autolysate agar (CYA) and malt extract agar (MEA) and incubated at 25 °C for 7 days. Colony diameter, colour of obverse and reverse, exudates and sclerotia were recorded. Conidial heads, vesicle shape, seriation, stipe texture and conidial ornamentation were examined in lactophenol cotton blue at $\times 400$ and $\times 1000$. Identification followed Klich (26), Pitt and Hocking (27) and Samson et al (6).

Molecular confirmation of species

Representative isolates of each morphotype were confirmed by sequencing. The ITS1-5.8S-ITS2 region was amplified with primers ITS1 and ITS4 (28), and part of the calmodulin gene with primers cmd5 and cmd6 (29), because calmodulin is the recommended secondary identification marker in *Aspergillus* (6). PCR products were purified and sequenced in both directions by a commercial sequencing service. Sequences were compared with type-strain sequences in GenBank by BLASTn, and an identity of $\geq 99\%$ with a type strain was accepted.

Screening of aflatoxigenic potential

Isolates of section *Flavi* were screened on coconut agar medium for blue fluorescence under UV light at 365 nm after 5 days at 28 °C (30). The ammonium hydroxide vapour test was used as a second screen; a pink to red colour

change on the colony reverse was scored as positive (31).

Genomic DNA extraction

Each isolate was grown in 50 mL potato dextrose broth at 28 °C for 3 days with shaking at 120 rpm. Mycelium was harvested by filtration, washed, blotted and ground in liquid nitrogen. DNA was extracted with a cetyltrimethylammonium bromide (CTAB) protocol (32). DNA purity and concentration were measured by spectrophotometry (A260/A280 between 1.8 and 2.0), integrity was checked on 0.8% agarose gel, and working stocks were diluted to 25 ng/ μ L.

RAPD-PCR amplification

Thirty-six isolates representing all species, grain sources and districts were selected for genotyping. Ten arbitrary decamer primers (Operon series A, B and C) were first screened on four representative isolates, and the five that gave clear, reproducible and polymorphic profiles were retained for the full panel (Table 3). Each 25 μ L reaction contained 1 $\times$ PCR buffer, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.4 μ M primer, 1 U Taq DNA polymerase and 50 ng template DNA (14). Thermal cycling consisted of initial denaturation at 94 °C for 5 min; 40 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min; and a final extension at 72 °C for 10 min. A no-template control was included in every run.

Products (10 μ L) were separated on 1.5% agarose gel in 1 $\times$ TBE buffer at 5 V/cm for about 2 h, alongside a 100 bp plus DNA ladder. Gels were stained with ethidium bromide (0.5 μ g/mL) and photographed under UV in a gel documentation system. To confirm reproducibility, every reaction was repeated twice with independently prepared mixes, and only bands present in both runs were scored.

Scoring and data analysis

Clear bands between 200 and 3000 bp were scored as present (1) or absent (0) for each isolate, producing a binary matrix. For each primer, the total number of bands (TNB),

number of polymorphic bands (NPB) and percentage polymorphism (PPB = $\text{NPB}/\text{TNB} \times 100$) were recorded. Polymorphic information content for dominant markers was calculated after Roldán-Ruiz et al (33), and resolving power after Prevost and Wilkinson (34):

$$\text{PIC}_i = 2f_i(1 - f_i)$$

$$R_p = \sum I_b, \quad I_b = 1 - (2 \times |0.5 - p|)$$

where f_i is the frequency of the amplified allele i , I_b is the informativeness of band b , and p is the proportion of isolates showing band b .

Pairwise genetic similarity was estimated with the Jaccard coefficient (35). A dendrogram was built by the unweighted pair group method with arithmetic mean (UPGMA) (36) in NTSYSpc version 2.1 (37). Fit of the dendrogram to the similarity matrix was tested by the cophenetic correlation coefficient (38), with significance assessed by the Mantel test (39) using 1000 permutations. Principal coordinate analysis (PCoA) was run on the same matrix.

Isolates were grouped by grain source and, separately, by species. For each group, the percentage of polymorphic loci, Nei's gene diversity (h) (40) and Shannon's information index (I) (41) were calculated in GenAlEx

version 6.5 (42). Analysis of molecular variance (AMOVA) partitioned the variation within and among groups, and significance was tested with 999 permutations (43). Differences in *Aspergillus* frequency among grains were tested with the chi-square test in IBM SPSS Statistics version 29 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered significant.

Ethical considerations

The study involved no human participants or animals. Grain samples were obtained with the permission of participating warehouse and market owners.

RESULTS

Fungal contamination and *Aspergillus* frequency

All 90 samples were contaminated with fungi. Mean grain moisture was 12.1% in wheat, 13.6% in maize and 11.4% in rice (Table 1). *Aspergillus* was recovered from 47 of 90 samples (52.2%). Its frequency was highest in maize (66.7%), followed by wheat (50.0%) and rice (40.0%), but the difference was not statistically significant ($\chi^2 = 4.36$, $df = 2$, $P = 0.113$). Maize also had the highest mean fungal count (2.4×10^4 CFU/g), followed by wheat and rice.

Table 1. Fungal contamination of stored wheat, maize and rice samples.

Grain	Samples (n)	Moisture, % (mean $\pm$ SD)	Total fungi, CFU/g (mean $\pm$ SD)	Samples with <i>Aspergillus</i> , n (%)	<i>Aspergillus</i> isolates (n)
Wheat	30	12.1 $\pm$ 1.4	$1.6 \times 10^4 \pm 0.5 \times 10^4$	15 (50.0%)	18
Maize	30	13.6 $\pm$ 1.7	$2.4 \times 10^4 \pm 0.7 \times 10^4$	20 (66.7%)	23
Rice	30	11.4 $\pm$ 1.2	$1.1 \times 10^4 \pm 0.4 \times 10^4$	12 (40.0%)	14
Total	90	12.4 $\pm$ 1.7	$1.7 \times 10^4 \pm 0.8 \times 10^4$	47 (52.2%)	55

A total of 55 *Aspergillus* isolates were obtained (one isolate per species per positive sample); because several samples yielded more than one species, the number of isolates exceeds the number of positive samples. Fifty-three were assigned to six species and two could not be identified to species level (Table 2). *A. flavus* was the most frequent species (Fr = 22.2%; RD = 36.4%), followed by *A. niger* (Fr = 18.9%; RD = 30.9%) and *A. parasiticus* (Fr = 7.8%; RD = 12.7%). ITS and calmodulin sequences of 12 representative isolates matched type strains at 99.0–99.8% identity, confirming the morphological identifications. Of the 27 section *Flavi* isolates (*A. flavus*, 20; *A. parasiticus*, 7), 15 (55.6%) were positive in both aflatoxigenicity screens.

Table 2. Isolation frequency (Fr) and relative density (RD) of *Aspergillus* species by grain.

Species	Wheat Fr, %	Wheat RD, %	Maize Fr, %	Maize RD, %	Rice Fr, %	Rice RD, %	Isolates (n)
<i>A. flavus</i>	26.7	44.4	36.7	47.8	3.3	7.1	20
<i>A. niger</i>	16.7	27.8	20.0	26.1	20.0	42.9	17
<i>A. parasiticus</i>	10.0	16.7	10.0	13.0	3.3	7.1	7
<i>A. ochraceus</i>	3.3	5.6	6.7	8.7	3.3	7.1	4
<i>A. fumigatus</i>	3.3	5.6	3.3	4.3	3.3	7.1	3
<i>A. terreus</i>	0.0	0.0	0.0	0.0	6.7	14.3	2
Unidentified <i>Aspergillus</i> spp.	0.0	0.0	0.0	0.0	6.7	14.3	2

Fr, percentage of the samples of each grain (n = 30) yielding the species; RD, percentage of all *Aspergillus* isolates from that grain. Overall Fr: *A. flavus* 22.2%, *A. niger* 18.9%, *A. parasiticus* 7.8%.

RAPD profiles and primer informativeness

Of the 10 primers screened, five gave reproducible, scorable profiles (**Table 3**). They amplified 58 bands (250–2700 bp), a mean of 11.6 bands per primer, of which 48 (82.8%) were polymorphic. Primer OPA-09 was the most informative (PIC = 0.44; Rp = 8.2) and OPB-07 the least (PIC = 0.22; Rp = 5.4). Four unique bands were detected; for example, a 950 bp band generated by OPA-09 occurred only in *A. flavus* isolates.

Table 3. Polymorphism generated by the selected RAPD primers.

Primer	Sequence (5'-3')	Total bands	Polymorphic bands	Polymorphism, %	Size range, bp	Unique bands	PIC	Rp
OPA-01	CAGGCC CTTC	12	10	83.3	300– 2400	1	0.35	7.1
OPA-03	AGTCAG CCAC	11	9	81.8	250– 2300	1	0.33	6.8
OPA-09	GGGTAA CGCC	13	12	92.3	350– 2700	2	0.44	8.2
OPB-07	GGTGAC GCAG	10	7	70.0	400– 2100	0	0.22	5.4
OPC-08	TGGACC GGTG	12	10	83.3	300– 2600	0	0.36	7.3
Total / mean	—	58	48	82.8	250– 2700	4	0.34	6.96

Genetic diversity by grain source and species

Genetic diversity was highest among maize isolates ($h = 0.31$; $I = 0.46$) and lowest among rice isolates ($h = 0.25$; $I = 0.38$) (**Table 4**). Among species, *A. flavus* was the most diverse ($h = 0.32$; $I = 0.47$). AMOVA attributed 82% of the total variation to differences within grain-source groups and 18% to differences among them ($\Phi_{PT} = 0.18$; $P = 0.018$) (**Table 5**), indicating weak but significant differentiation by commodity.

Table 4. Genetic diversity indices of *Aspergillus* isolates by grain source and by species.

Group	Isolates (n)	Polymorphic loci, %	Nei's gene diversity, h ($\pm$ SE)	Shannon index, I ($\pm$ SE)
Wheat	12	79.3	0.28 ± 0.03	0.42 ± 0.04
Maize	14	81.0	0.31 ± 0.03	0.46 ± 0.04
Rice	10	72.4	0.25 ± 0.04	0.38 ± 0.05
<i>A. flavus</i>	14	75.9	0.32 ± 0.03	0.47 ± 0.04
<i>A. niger</i>	11	69.0	0.29 ± 0.04	0.43 ± 0.05
All isolates	36	82.8	0.34 ± 0.02	0.50 ± 0.03

Table 5. Analysis of molecular variance (AMOVA) among and within grain-source groups.

Source of variation	df	Sum of squares	Mean squares	Estimated variance	Variation, %	<i>P</i>
Among grain sources	2	18.00	9.00	0.55	18	0.018
Within grain sources	33	82.50	2.50	2.50	82	—
Total	35	100.50	—	3.05	100	—

$\Phi_{PT} = 0.18$. *P* values were obtained from 999 random permutations. Variance components were estimated from mean squares with $n_0 = 11.89$.

DISCUSSION

This study showed that *Aspergillus* contaminated 52.2% of stored wheat, maize and rice samples from Haditha, Hit, Ramadi and Rutba (Anbar Governorate, Iraq), and that the isolates were genetically diverse. The dominance of *A. flavus* agrees with recent regional data from Egypt, in which section *Flavi* was recovered from all barley and maize samples collected from markets in Qena (8), and with Iraqi surveys in which *A. flavus* and *A. niger* were the main *Aspergillus* species in stored wheat and maize (10–12). Maize carried the highest fungal load and the highest *Aspergillus* frequency, consistent with its higher moisture content and its known susceptibility to aflatoxigenic fungi (2,9), although the difference among grains did not reach statistical significance with 30 samples per commodity. The presence of presumptively aflatoxigenic isolates in heavily contaminated stored maize is a direct food-safety concern, because aflatoxin B1 is the main mycotoxin reported in maize from the region (9,12), and it has also been detected in rice marketed in Iraq (13).

The five RAPD primers revealed 82.8% polymorphism, slightly below earlier reports. *A. flavus* from Saudi wheat, maize and barley showed high RAPD variability (19–21), and 14 primers produced 99.4% polymorphic bands among 24 Iraqi *A. niger* isolates (24). Such differences partly reflect the number of primers used and whether one or several species are analysed together. In Egypt, RAPD distinguished 12 soil *Aspergillus* species and showed similarity above 80% between *A. terreus* and *A. aculeatus* (22), while Mohamad reported wide similarity ranges among local *A. flavus* isolates (23). The mean PIC of 0.34 indicates that the selected primers were moderately to highly informative, since 0.5 is the theoretical maximum for dominant markers (33).

Clustering grouped isolates mainly by species, which supports earlier work showing that RAPD patterns separate *Aspergillus* species reliably and that some primers yield species-specific bands (16–18). Within species, isolates did not cluster by grain source or district, as also reported for *A. flavus* from Saudi cereals (19–21). Consistently, AMOVA placed most of the

variation within grain-source groups, with only modest differentiation among commodities. This suggests that conidia move freely between commodities through shared stores, transport and trade, as expected for a largely clonal fungus whose populations are shaped by vegetative compatibility groups and occasional recombination (44).

RAPD profiles did not separate aflatoxigenic from non-aflatoxigenic section *Flavi* isolates in this dataset, in agreement with earlier RAPD and ISSR studies (19–21). Loss of toxigenicity often results from deletions or point mutations in the aflatoxin gene cluster, which anonymous markers are unlikely to target (45,46). Gene-specific PCR of *aflR*, *aflD* and *aflM*, as applied to stored grains in Egypt (8), is therefore a necessary complement.

The high diversity has practical consequences. Section *Flavi* now contains many cryptic aflatoxigenic species (7,47,48), and a genetically varied population is more likely to include highly toxigenic or clinically relevant strains (4). Monitoring storage fungi at the genotype level can thus help identify stores with high-risk populations and guide drying, aeration and fumigation decisions (2). Non-aflatoxigenic genotypes identified in such surveys are also candidates for biological control (44).

This study has limitations. RAPD markers are dominant and sensitive to reaction conditions; we minimised this by duplicate amplifications and by scoring only reproducible

bands. Only 36 isolates were genotyped, and species other than *A. flavus* and *A. niger* were too few for separate diversity estimates. Sampling covered four districts of a single governorate and a single storage season, so seasonal and regional effects could not be tested. Toxigenicity was screened by cultural methods rather than quantified by HPLC. Future work should combine codominant markers, such as SSRs or multilocus sequencing (25), with toxin quantification across seasons and regions.

CONCLUSION

Stored wheat, maize and rice in Haditha, Hit, Ramadi and Rutba, Anbar Governorate, Iraq, harboured a genetically diverse *Aspergillus* community dominated by *A. flavus*, and more than half of the section *Flavi* isolates were presumptively aflatoxigenic. Five RAPD primers produced 82.8% polymorphism and grouped isolates mainly by species, with only modest differentiation among grain sources. RAPD-PCR is therefore a rapid, low-cost tool for baseline surveillance of storage *Aspergillus* populations, but it should be combined with toxin-gene PCR and codominant markers to assess food-safety and public-health risk.

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