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RESEARCH ARTICLE

Genetic and chemotypic diversity of two lineages of *Aspergillus flavus* isolated from maize seeds of different agroclimatic niches of India

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Abstract

Maize is susceptible to colonization by *Aspergillus flavus,* which is the major aflatoxins producing fungus. Aflatoxins are fungal secondary metabolites that have been reported to adversely affect normal cell metabolism and seed germination. In this study, 106 maize seed samples were sampled across 13 states throughout India, and were screened for incidences of fungal infection. Among the seed samples collected across India nearly all maize seed samples were found to be infected with *Aspergillus*. All morphologically confirmed isolates belonging to *Aspergillus* section *Flavi* were subjected to various aflatoxin, biochemical, physiological and genotype assays, as well as phylogenetic analyses. Among 60 isolates tested, eight were non-aflatoxigenic in nature as determined by icELISA, and seven of those eight isolates showed absence of one or more aflatoxin cluster genes in PCR assay. The morphological, physiological and biochemical characterizations failed to show variation based on their toxigenicity. The phenetic and molecular genetic diversity could be observed based on RAPD and ISSR analyses and little isolate diversity could be determined based on geography or aflatoxigenicity. An inferred ITS sequence phylogeny segregated isolates into only two lineage groups: a large group I (including Ia and Ib) and small group II contained *A. flavus* isolates closely related to *A. parasiticus.* The current study highlights inherent diverse *A. flavus* infections, exhibiting a range of aflatoxin concentrations, that are present in maize seeds and they are important because of the potential threat to agricultural production and consumption.

Keywords Aflatoxin · *Aspergillus flavus* · mPCR · Genetic diversity · Maize · UPGMA · GPS coordinates

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Abbreviations

RAPD	Random amplified polymorphic DNA
ISSR	Inter simple sequence repeats
ITS	Internal transcribed spacer regions
UPGMA	Unweighted pair group method with arithmetic mean
NTSYS-pc	Numerical taxonomy and multivariate analysis system

Background

Maize (*Zea mays* Link) is one of the most important food staples, not only for humans, but also for animals, and is equally important as raw material for the manufacture of many industrial products (Nayaka et al. 2009; White 1999). India has 3.4% of its agricultural area covered in maize cultivation, and now ranks sixth in world production (Rasul 2016). Maize is grown in most parts of the India, ranging from extreme humid to sub-humid regions and most popularly in north-eastern and western regions (Joshi 2005). Even though hybrid seeds are used in most maize growing regions, traditional farmers still depend on their own saved seeds for subsequent plantings (Aiyaz et al. 2015a; Morris et al.1998; Mudili et al. 2014; Rauniyar and Goode 1992). Maize seeds are rich in carbohydrates, which may attract a wide range of fungal pathogens during pre- and post-harvest storage, and from a seed stock perspective *Aspergillus*-infected seeds often exhibit poor germination, of seed leading to poor yield and growth (Aiyaz et al. 2015b; Solorzano and Malvick 2011). It is possible that compounds produced by these fungi (including toxic metabolites) have the potential to adversely affect normal cell metabolism and seed germination (Aiyaz et al. 2015c; Nayaka et al. 2008; Prasad et al. 1997). Likewise, it is demonstrated that, among the fungal pathogens, moulds such as *Aspergillus* are able to thrive under adverse conditions of temperature (13–37 °C) and moisture (approximate minimum aw 0.82) (Lawley et al. 2008). Aflatoxin (AF) biosynthesis exists in several *Aspergillus* species, but the most widely known and researched of those species is *A. flavus* (Divakara et al. 2015; Godet and Munaut 2010). Aflatoxin B1 is considered has Class 1 human carcinogen by International Agency for Research (Anttila et al. 2002), hence, aflatoxin levels food products are carefully examined and regulated in most countries. In developed and developing countries most of them have established and proposed regulation for controlling aflatoxins in food products (Anttila et al. 2002). Production of AF involves at least 23 enzymatic reactions orchestrated by 25 genes, representing a well-defined gene cluster located in a 75 kb telomeric region of chromosome 3 (Carbone et al. 2007; Yu et al. 2004). Few *A. flavus* in nature lack the ability to synthesis aflatoxins due to either mutation in, or deletion of, any of the genes within the cluster (Criseo et al. 2008; Kiyota et al. 2011).

Conventional methods used for species identification of seed-borne fungal pathogens are mostly based on fungal morphology such as colony diameter, color and texture, as well as the size and texture of conidia and conidiophore structures (Chandra et al. 2013; Klich 2002). These fungi are also known to show variation in optimum pH, temperature, and required carbon and nitrogen sources (Chellapandi and Jani 2009). Differing or altering the carbon sources are known to alter secondary metabolite production which includes mycotoxins (Paranagama et al. 2007). Fungi produce various extracellular enzymes such as lipase, protease, cellulase, pectinase and amylase with roles in primary metabolism, plant pathogenicity and also in mycotoxin production (Alam et al. 2009; Fakhoury and Woloshuk, 1999; Ortega et al. 2013; Venkataramana et al. 2015). The phylogenetic nature of *A. flavus* is genetically complex with the presence of several cryptic species which at times makes species identification difficult (Geiser et al. 2000).

Fungal species identification has been made simple and fast, with the advancement in the field of molecular technique (Venkataramana et al. 2012; Vitale et al. 2011). Molecular technique like amplified fragment length polymorphism (AFLP), fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplification of the internal transcribed spacer region (ITS), restriction inter-simple sequence repeats (ISSR), and single-strand conformation polymorphism (SSCP), all of which are auxiliary tools in traditional methods (Nitschke et al. 2009; Dendis et al. 2003; Kumeda and Asoa 2001).

The objectives of the present study were to investigate the occurrence of aflatoxin production associated with fungal infection in maize seed used for planting, and molecular analysis of the genetic diversity of *A. flavus* isolated from infected maize seed.

Methods

Sample collection

A survey was conducted across maize growing regions of India during the years of 2008–2011, a total of 106 seed samples (Table 1) were collected from agricultural Universities, seed corporations, research institutes and farm seed stock, irrespective of storage conditions. A minimum of 2.5 kg of maize seeds were collected per sample and labeled. The sampling points were documented using a Garmin handheld GPS 76CSx recorder and collection points were located at various agro-climatic regions across 13 states in India (Gopal et al. 2009). The GPS coordinates were converted to

Table 1 Seed samples with incidences of *Aspergillus* infection

Sample code	GPS Coordinates	*Aspergillus* spp. infection*
Karnataka		
M 1	13°10′ N & 76°18′ E	85.02 ± 0.04
M 2	13°10′ N & 76°18′ E	03.97 ± 0.04
M 3	13°10′ N & 76°18′ E	30.00 ± 0.08
M 4	13°10′ N & 76°18′ E	41.97 ± 0.08
M 5	13°10′ N & 76°18′ E	19.97 ± 0.04
M 6	13°10′ N & 76°18′ E	30.07 ± 0.11
M 7	14°32′ N & 75°49′ E	24.95 ± 0.06
M 8	14°32′ N & 75°49′ E	05.05 ± 0.06
M 9	14°32′ N & 75°49′ E	19.97 ± 0.08
M 10	15°13′ N & 75°33′ E	03.00 ± 0.04
M 11	14°30′ N & 75°51′ E	16.05 ± 0.06
M 12	14°21′ N & 76°36′ E	28.02 ± 0.04
M 13	14°28′N & 76°05′ E	25.05 ± 0.06
M 14	15°13′ N & 75°34′ E	05.05 ± 0.06
M 15	13°10′ N & 76°18′ E	01.97 ± 0.04
M 16	13°48′ N & 76°18′ E	02.05 ± 0.06
M 17	14°58′ N & 75°19′ E	18.02 ± 0.08
M 18	14°58′ N & 75°19′ E	04.05 ± 0.06
M 19	12°19′ N & 76°33′ E	03.00 ± 0.04
M 20	12°38′ N & 76°02′ E	02.00 ± 0.04
M 21	12°38′ N & 76°02′ E	16.00 ± 0.04
M 22	12°27′ N & 76°23′ E	07.02 ± 0.04
M 23	12°29′ N & 76°54′ E	04.92 ± 0.04
M 24	12°38′ N & 76°02′ E	10.05 ± 0.06
M 25	12°38′ N & 76°02′ E	15.97 ± 0.08
M 26	12°38′ N & 76°02′ E	60.07 ± 0.11
M 27	12°38′ N & 76°02′ E	19.97 ± 0.08
M 28	13°18′ N & 76°13′ E	06.00 ± 0.04
M 29	12°19′ N & 76°34′ E	10.95 ± 0.06
M 30	11°48′ N & 76°42′ E	16.05 ± 0.06
M 31	12°20′ N & 76°52′ E	10.05 ± 0.06
M 64	13°04′ N & 77°35′ E	55.02 ± 0.08
M 80	15°27′ N & 75°01′ E	30.00 ± 0.07
M 81	15°27′ N & 75°01′ E	51.02 ± 0.06
M 84	13°04′ N & 77°35′ E	48.00 ± 0.07
M 85	13°04′ N & 77°35′ E	64.05 ± 0.06
M 86	13°04′ N & 77°35′ E	07.05 ± 0.06
M 87	13°04′ N & 77°35′ E	00.07 ± 0.04
M 88	26°54′ N & 75°48′ E	21.05 ± 0.06
Tamil Nadu		
M 32	10°59′ N & 76°56′ E	24.87 ± 0.29
M 33	10°59′ N & 76°56′ E	20.00 ± 0.04
M 34	10°59′ N & 76°56′ E	24.95 ± 0.02
M 35	10°59′ N & 76°56′ E	22.22 ± 0.22
M 36	10°59′ N & 76°56′ E	24.00 ± 0.10
M 37	10°59′ N & 76°56′ E	05.02 ± 0.04
M 38	10°28′ N & 79°16′ E	11.00 ± 0.04
M 39	10°28′ N & 79°16′ E	23.92 ± 0.14
M 40	11°24′ N & 78°31′ E	16.05 ± 0.06
M 41	11°24′ N & 78°31′ E	25.02 ± 0.04
Andhra Pradesh		
M 43	17°11′ N & 78°23′ E	47.05 ± 0.06
M 44	17°11′ N & 78°23′ E	69.97 ± 0.04
M 45	17°11′ N & 78°23′ E	00.97 ± 0.12
M 46	17°11′ N & 78°23′ E	01.97 ± 0.04
M 47	17°11′ N & 78°23′ E	05.97 ± 0.20
M 48	18°00′ N & 79°33′ E	38.00 ± 0.04
M 49	18°00′ N & 79°33′ E	33.97 ± 0.04
M 50	18°00′ N & 79°33′ E	34.02 ± 0.06
M 51	18°00′ N & 79°33′ E	29.77 ± 0.22
M 52	16°18′ N & 80°24′ E	28.00 ± 0.04
M 53	16°18′ N & 80°24′ E	40.05 ± 0.06
M 54	16°18′ N & 80°24′ E	28.00 ± 0.04
M 55	16°18′ N & 80°24′ E	00.02 ± 0.02
M 56	15°40′ N & 78°02′ E	25.19 ± 0.26
M 57	15°40′ N & 78°02′ E	11.07 ± 0.04
M 58	15°40′ N & 78°02′ E	14.00 ± 0.04
M 59	15°40′ N & 78°02′ E	19.97 ± 0.02
M 60	15°40′ N & 78°02′ E	45.25 ± 0.47
M 65	16°59′ N & 81°50′ E	20.22 ± 0.25
M 66	16°59′ N & 81°50′ E	13.02 ± 0.04
M 67	16°59′ N & 81°50′ E	14.97 ± 0.02
M 73	15°50′ N & 78°02′ E	00.25 ± 0.25
M 104	16°18′ N & 80°24′ E	15.02 ± 0.04
Maharashtra		
M 91	18°30′ N & 73°50′ E	30.00 ± 0.40
M 92	18°30′ N & 73°50′ E	37.25 ± 0.32
M 61	22°45′ N & 75°45′ E	23.97 ± 0.06
M 62	22°45′ N & 75°45′ E	34.50 ± 0.64
Gujarat		
M 42	22°32′ N & 72°56′ E	36.82 ± 0.53
Rajasthan		
M 68	24°39′ N & 74°01′ E	36.85 ± 0.31
M 69	24°39′ N & 74°01′ E	39.52 ± 0.30
M 70	24°39′ N & 74°01′ E	87.00 ± 0.91
M 71	24°39′ N & 74°01′ E	13.00 ± 0.04
M 72	24°39′ N & 74°01′ E	30.37 ± 0.55
M 74	24°39′ N & 74°01′ E	41.72 ± 0.24
M 75	24°39′ N & 74°01′ E	9.92 ± 0.04
M 82	28°07′ N & 73°02′ E	15.00 ± 0.04
M 83	13°04′ N & 77°35′ E	00.05 ± 0.02
M 89	26°54′ N & 75°48′ E	37.17 ± 0.24
M 90	26°54′ N & 75°48′ E	40.02 ± 0.04
M 93	28°07′ N & 73°02′ E	08.00 ± 0.04
M 95	24°39′ N & 74°01′ E	37.00 ± 0.04
M 96	24°39′ N & 74°01′ E	39.90 ± 0.13
M 97	24°39′ N & 74°01′ E	34.05 ± 0.06

Table 1 (continued)

Sample code	GPS Coordinates	*Aspergillus* spp. infection*
M 98	24°39′ N & 74°01′ E	07.87 ± 0.04
M 99	24°39′ N & 74°01′ E	07.25 ± 0.75
Haryana		
M 79	15°27′ N & 75°01′ E	09.75 ± 0.47
West bengal		
M 63	22°32′ N & 88°21′ E	20.27 ± 0.58
M 94	22°32′ N & 88°21′ E	22.00 ± 0.04
Uttarakhand		
M 100	28°38′ N & 77°09′ E	15.97 ± 0.04
M 101	28°38′ N & 77°09′ E	12.97 ± 0.02
M 102	28°38′ N & 77°09′ E	15.72 ± 0.24
Manipur		
M 76	24°49′ N & 93°54′ E	11.92 ± 0.16
M 77	24°49′ N & 93°54′ E	40.95 ± 0.15
M 78	29°08′ N & 75°44′ E	22.02 ± 0.04
New Delhi		
M 103	16°18′ N & 80°24′ E	04.92 ± 0.04
M 105	28°38′ N & 77°09′ E	09.97 ± 0.02
Himachal Pradesh		
M 106	31°02′ N & 76°41′ E	14.95 ± 0.02

*Mean values of four independent replicates. The ± symbol indicates standard error

'shapefiles' using Quantum GIS Lisboa where the attributes were layered to generate a study area (Fig. 1).

Assessment of fungal contamination

All maize seed samples were examined by standard blotter method (SBM) (ISTA 2005). To assess seed-borne fungi, seeds from each sample (Table 1) maize seeds were placed on wet filter paper in sterile pertiplates. Plated seeds were kept moist by frequently wetting filter paper with sterile distill water in two days interval. Fungal contamination was evaluated across all the seeds after seven days. Fungal identification was done based by using stereomicroscope under different magnifications and identified based on the morphological and colony characters (Singh et al. 1991). Isolates tentatively determined belonging to *Aspergillus* section *Flavi* were maintained as pure cultures on potato dextrose agar (PDA) medium at 4 °C and used for further studies.

Characterization of *Aspergillus flavus* isolates

A representative sample of 60 *Aspergillus flavus* isolates was characterized using various optimal media such as Potato Dextrose Agar (PDA), *Aspergillus Flavus* Parasiticus Agar (AFPA), Yeast Extract Sucrose (YES) agar and Czapek dox (CZ) agar, and Physiological characterization for all isolates involved growing them on PDA with modified pH levels of 4, 6, 8, 10 and 12 using tartaric acid and NaOH. Additionally, the ability of these *Aspergillus* isolates to grow in PDA at different temperatures (4, 15, 30 and 40 °C) was analyzed.

The ability of isolates to produce extracellular enzymes like amylase, lipase, protease, cellulase and pectinase was tested using culture plate assays (Sunitha et al. 2013). The isolates were point-inoculated in each medium type and their colony characteristics were observed after 7 days after incubation. High-Performance Thin Layer Chromatography (HPTLC) analysis was also performed for all isolates to determine their aflatoxin-producing potential (Criseo et al. 2001). Further aflatoxins were quantified indirect competitive (ic) ELISA (Reddy et al. 2009; Hariprasad et al. 2013).

Molecular identification of *Aspergillus flavus* isolates

All 60 *Aspergillus* isolates were subjected to a molecular confirmation of their preliminary species identification. DNA was extracted from the isolates using a Hi PurA™ Plant Genomic DNA Miniprep Purification Spin Kit (Hi media, India) according to manufacturer's instructions. A genera-specific PCR assay was then performed for confirmation of *Aspergillus* species using Aspergillopepsin primers PEPO1 (CGACGTCTACAAGCCTTCTGGAAA) and PEPO2 (CAGCAGACCGTCATTGTTCTTGTC), PCR amplification was carried and genra specific identification of *Aspergillus* was done presence of specific band when visualized under UV transilluminator (Logotheti et al. 2009; Priyanka et al. 2013). Furthermore, these isolates were confirmed at the molecular species level according White et al. (1990). Multiplex PCR (mPCR) reactions were performed with primers for specific AF cluster genes selected: *aflR, ordA, norA, omtA,* and *ver1* (Table 2) (Yin et al. 2009).

Phylogenetic and phenetic analyses

Sequences obtained from the ITS regions of all isolates, along with an outlier sequence of *Aspergillus parasiticus* (EU982020.1) from NCBI, were aligned using ClustalW version 1.7 (Thompson et al. 1994). The resulting phylogenetic tree was inferred by maximum likelihood method using the Mega 5.0 program (Tamura et al. 2011). Genetic distance was calculated using the Kimura 2-parameter model (Kimura et al. 1980).

RAPD analysis was performed by selecting 20 random decamer primers (Kini et al. 2002). These RAPD primers were first evaluated for their proven ability to amplify genomic DNA fragments for a few of our *Aspergillus* isolates. A total of 15 primers were used (Table 3). The PCR reaction was carried and amplified products were then separated in a 1.5% agarose gel stained with ethidium bromide and compared with Mass Ruler Express DNA ladder (Kini

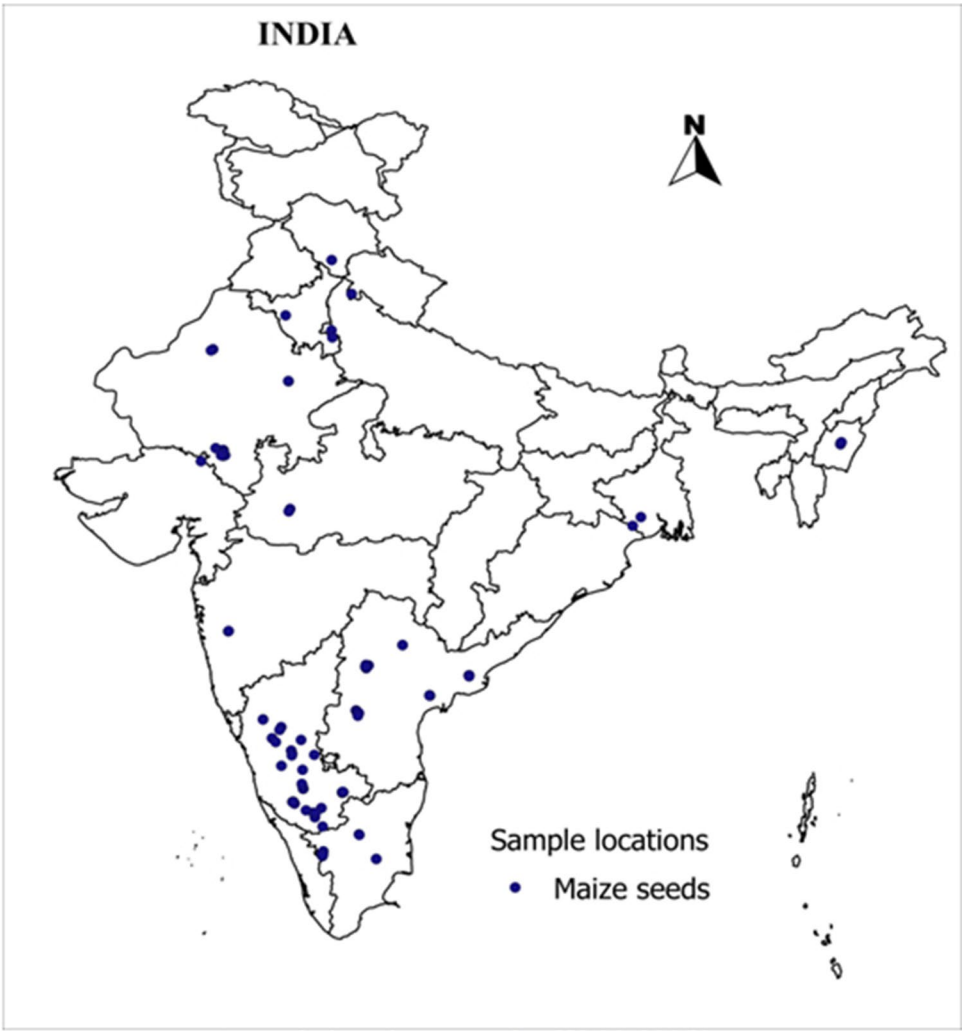

Fig. 1 Map of India with sampling locations marked as blue dots. Sampling sites represent various agro-climatic regions across 13 states

Table 2 Target aflatoxin cluster gene primers used in this study with expected amplicon size

Primer name	Primer sequence	Target region	Amplicon size (bp)
aflR-F	TGAGAAAGGGGACGCTGGAT	*aflR*	873
aflR-R	CAATCGAATCAACCACCACA		
ver1-F	ACCACCGTTTAGATGGCAAA	*ver1*	472
ver1-R	AGAGCTGGTCAGGATAATCCG		
omtA-F	CAGGATATCATTGTGGACGG	*omtA*	594
omtA-R	CTCCTCTACCAGTGGCTTCG		
ordA-F	TATTCTGGGCGAAGCATCAA	*ordA*	1154
ordA-R	CAGAGTAGTTGGTCCCACGA		
norA-F	CCTTATGCCTGGGAACGAT	*norA*	399
norA-R	TTCGCATCACTTCCTCCACA		

et al. 2002). ISSR analysis, was carried out by 21 different set of primers (Table 4) and band visualization was done according to Divakara et al. (2015).

Unambiguous ISSR and RAPD fragments were scored for the presence (1) or absence (0) of ISSR patterns in each of the 60 isolates. In some cases, isolates failed to amplify

Table 3 Primer sequences used for RAPD analysis

ISSR primer	Sequence motif	Annealing temp (°C)	Monomorphic bands[a]	Polymorphic bands[a]	Polymorphism (%)[a]
OPA1	CAGGCCCTTC	40	2	6	75
OPA3	AGTCAGCCAC	40	0	5	100
OPA4	AATCGGGCTG	40	3	7	70
OPA5	AGGGGTCTTG	40	4	5	55
OPA7	GAAACGGGTG	40	2	7	77
OPA8	GTGACGTAGG	40	2	6	75
OPA9	GGGTAACGCC	40	1	8	88
OPA10	GTGATCGCAG	40	4	4	50
OPA11	CAATCGCCGT	40	3	9	75
OPA12	TCGGCGATAG	40	1	3	75
OPA13	CAGCACCCAC	40	2	10	83
OPA14	TCTGTGCTGG	40	2	4	66
OPA15	TTCCGAACCC	40	1	7	87.5
OPA16	AGCCAGCGAA	40	4	5	55
OPA17	GACCGCTTGT	40	2	4	66

[a]Results based on amplification of all primers across 60 *Aspergillus* isolates

Table 4 Primer sequences used for ISSR analysis

ISSR primer	Sequence motif	Annealing temp °C	Monomorphic bands[a]	Polymorphic bands[a]	Polymorphism (%)[a]
ISSR02	$(CT)_7AC$	40	1	4	80
ISSR03	$(CT)_7GC$	43	1	5	83.3
ISSR04	$(CA)_6AC$	43	0	3	100
ISSR05	$(CA)_6GT$	43	0	9	100
ISSR06	$(CA)_6AG$	43	2	3	60
ISSR07	$(CA)_6GC$	43	1	7	87.5
ISSR09	$(GT)_6GG$	40	1	5	83.3
ISSR10	$(GA)_6CC$	40	3	6	66.6
ISSR12	$(CAC)_3GC$	40	0	4	100
ISSR13	$(GAG)_3GC$	46	3	6	66.6
ISSR14	$(CTC)_3GC$	43	0	11	100
ISSR16	$(GA)_9T$	43	2	3	60
ISSR19	$(GACA)_4$	43	1	7	87.5
ISSRA1	$(GA)_8T$	46	2	4	66.6
ISSRA2	$(AC)_8T$	48	1	3	75
ISSRA3	$(AG)_7C$	41	2	4	66.6
ISSRA6	$(CCA)_6$	68	0	10	100
ISSRA7	$(AG)_8G$	44	0	8	100
ISSRA9	$(ATG)_6$	46	1	5	83.3
ISSRA10	$(GA)_8T$	46	1	2	66.6

[a]Results based on amplification of all primers across 60 *A. flavus* isolates

any product with certain primers. In these cases, missing data was scored as "?". Those isolates exhibiting similar banding patterns were referred to as monomorphic, whereas those that exhibited a different pattern were referred to as polymorphic (Rohlf et al. 1998). The data produced by the RAPD and ISSR methods were analysed (Felsenstein et al. 1993). The consistency of the phylogenetic trees drawn by the NJ method was explored by bootstrap resampling using

the SEQBOOT program (PHYLIP 3.5) 100 replicates, of random seed 81 for RAPD, ISSR and combined data.

Results

Fungal contamination of seed samples

Of the 106 seed samples, the overall incidence of infection by *Aspergillus* section *Flavi* was 95%, although the occurrence of infection within the seeds of each 2.5 kg sample ranged from 1–88% (Table 1). Highest seed-borne incidence by *Aspergillus* section *Flavi* was observed in sample M81 which showed to have a contamination rate of 85%, and five seed samples (M37, M55, M73, M83 and M87) were free from any inherent *Aspergillus* infection (Table 1).

Characterization of *Aspergillus flavus* isolates

All 60 *Aspergillus flavus* isolates showed fast growth and early sporulation on PDA media, followed by CZ and YES, and diminished growth rate and sporulation were observed on AFPA medium. The isolates studied showed better growth at pH 8, and the least observed growth was at pH 4. The optimum temperature for the growth of the *Aspergillus flavus* isolates tested was 30°C, and least amount of growth was observed at 4°C.

All studied isolates were positive for extracellular amylase, cellulase and lipase enzyme production (Table 5). Among studied isolates for protease production, only two *Aspergillus* isolates (UOMMAF-11 and UOMMAF-25) were negative for production of the enzyme. Isolates UOMMAF-15, UOMMAF-37 and UOMMAF-54 were found to be negative for pectinase production. Based on the use of HPTLC, 27 of the 60 *Aspergillus* isolates exhibited production of both aflatoxins B1 and B2, 20 isolates produced only AF B1, five isolates produced only AF B2, and the remaining eight isolates (UOMMAF-2, UOMMAF-11, UOMMAF-26, UOMMAF-30, UOMMAF-33, UOMMAF-57, UOMMAF-64 and UOMMAF-71) were determined to be non-aflatoxigenic (Table 6). The icELISA confirmed the non-aflatoxigenic phenotype for seven of these eight isolates. Isolate UOMMAF-71, which appeared negative in HPTLC, exhibited the production of AF in the icELISA. All other isolates tested positive for AF production in icELISA, correlating positively with HPTLC (Table 6, Supplementary Table).

Molecular investigation of *Aspergillus flavus* isolates

PCR assays of genomic DNA with Aspergillopepsin primers were able to produce an expected amplicon fragment of about 200 bp in all isolates; therefore, the identities of the 60 morphologically identified isolates were confirmed

Table 5 Isolates of *Aspergillus* used in this study and their respective enzyme production

Isolate	Seed Sample	Extracellular enzymes produced[a]
Karnataka		
UOMMAF-1	M 1	+/+/+/+/+
UOMMAF-2	M 2	+/+/+/+/+
UOMMAF-3	M 4	+/+/+/+/+
UOMMAF-4	M 7	+/+/+/+/+
UOMMAF-5	M 9	+/+/+/+/+
UOMMAF-6	M 10	+/+/+/+/+
UOMMAF-7	M 11	+/+/+/+/+
UOMMAF-8	M 12	+/+/+/+/+
UOMMAF-9	M 13	+/+/+/+/+
UOMMAF-11	M 16	+/+/+/+/–
UOMMAF-13	M 15	+/+/+/+/+
UOMMAF-14	M 17	+/+/+/+/+
UOMMAF-15	M 19	+/+/+/–/+
UOMMAF-16	M 21	+/+/+/+/+
UOMMAF-18	M 23	+/+/+/+/+
UOMMAF-19	M 26	+/+/+/+/+
UOMMAF-20	M 29	+/+/+/+/+
UOMMAF-49	M 30	+/+/+/+/+
UOMMAF-65	M 64	+/+/+/+/+
UOMMAF-66	M 81	+/+/+/+/+
UOMMAF-67	M 86	+/+/+/+/+
Tamil Nadu		
UOMMAF-23	M 32	+/+/+/+/+
UOMMAF-24	M 33	+/+/+/+/+
UOMMAF-25	M 36	+/+/+/+/–
UOMMAF-26	M 38	+/+/+/+/+
UOMMAF-27	M 39	+/+/+/+/+
UOMMAF-28	M 40	+/+/+/+/+
UOMMAF-29	M 41	+/+/+/+/+
Andhra Pradesh		
UOMMAF-32	M 43	+/+/+/+/+
UOMMAF-33	M 44	+/+/+/+/+
UOMMAF-37	M 48	+/+/+/–/+
UOMMAF-38	M 51	+/+/+/+/+
UOMMAF-43	M 52	+/+/+/+/+
UOMMAF-44	M 54	+/+/+/+/+
UOMMAF-50	M 56	+/+/+/+/+
UOMMAF-52	M 60	+/+/+/+/+
UOMMAF-57	M 65	+/+/+/+/+
UOMMAF-68	M 67	+/+/+/+/+
UOMMAF-73	M 104	+/+/+/+/+
Gujarat		
UOMMAF-30	M 42	+/+/+/+/+
Madhya Pradesh		
UOMMAF-46	M 61	+/+/+/+/+
UOMMAF-47	M 62	+/+/+/+/+

Table 5 (continued)

Isolate	Seed Sample	Extracellular enzymes produced[a]
West Bengal		
UOMMAF-48	M 94	+/+/+/+/+
Rajasthan		
UOMMAF-53	M 68	+/+/+/+/+
UOMMAF-54	M 70	+/+/+/–/+
UOMMAF-55	M 71	+/+/+/+/+
UOMMAF-56	M 72	+/+/+/+/+
UOMMAF-58	M 74	+/+/+/+/+
UOMMAF-59	M 75	+/+/+/+/+
UOMMAF-60	M 82	+/+/+/+/+
UOMMAF-61	M 90	+/+/+/+/+
UOMMAF-69	M 95	+/+/+/+/+
UOMMAF-70	M 96	+/+/+/+/+
UOMMAF-71	M 97	+/+/+/+/+
Manipur		
UOMMAF-62	M 77	+/+/+/+/+
Haryana		
UOMMAF-63	M 79	+/+/+/+/+
UOMMAF-64	M 93	+/+/+/+/+
Uttara Khand		
UOMMAF-72	M 100	+/+/+/+/+
UOMMAF-74	M 102	+/+/+/+/+
Himachal Pradesh		
UOMMAF-75	M 106	+/+/+/+/+

[a]Presence (+) or absence (–) of extracellular enzymes: amylase/cellulase/lipase/pectinase/protease

to *Aspergillus* genus (Fig. 2). Further, PCR amplification with ITS primers produced an expected amplicon fragment of approximately 600 bp (Fig. 3), and their respective sequences were then BLAST-confirmed as *Aspergillus flavus* based on 94–100% identity and genBank accession numbers were obtained (Table 6).

Multiplex PCR results are shown in Fig. 4 and Table 6. Amplicon fragments of the *norA, ver1, omtA, aflR,* and *ordA* genes can be visualized at 399 bp, 472 bp, 594 bp, 873 bp and 1156 bp, respectively. Of the 60 isolates examined, 54 showed a pentaplex band pattern indicating the presence of the five target genes from the AF biosynthesis pathway. Non-aflatoxigenic isolates UOMMAF-2, UOMMAF-11 UOMMAF-26, UOMMAF-33, UOMMAF-57 and UOMMAF-64 showed banding patterns in which one or more bands were absent. Isolate UOMMAF-30; however, which was also found to be negative for AF production in both HPTLC and icELISA, exhibited the presence of all five bands in mPCR (Fig. 4).

Phylogenetic and phenetic analyses

A phylogenetic tree, inferred by the maximum likelihood method, and based on the ITS region, segregated the 60 isolates into two clades (Fig. 5). Clade A divided into two subclades supported by a bootstrap value of 62 which consists of the *A. flavus* lineage I isolates, whereas lineage II clade B consists of the out-group taxa *A. parasiticus* (EU902020.1) and three isolates of *A. flavus* lineage II (UOMMAF-2, UOMMAF-67 and UOMMAF-68), which were isolated from seed samples collected in Karnataka state and Andhra Pradesh. Both branches in the maximum likelihood method tree were strongly supported by high bootstrap values of > 90 that are labeled at nodal regions (Fig. 5).

The 15 selected decamer RAPD primers generated 123 unambiguous DNA bands; of which, 90 were polymorphic with an average of 6.0 polymorphic fragments per primer (Table 3). A phenetic UPGMA tree was inferred using dice similarity based on the coefficient dendrogram of RAPD data. The 60 *Aspergillus* isolates showed an ancestral bifurcation into two lineages, with isolate UOMMAF-28 being the only representative isolate for one of those lineages (noted as II in Fig. 6), and scoring a similarity coefficient of 0.44. Lineage I included the remaining isolates for which the similarity coefficient was 0.61 despite the high number of bifurcating branches observed (Fig. 6). In the phylogenetic NJ consensus tree (not shown) six clades were supported by bootstrap values higher than 50%. In one of them both isolates UOMMAF-32 and UOMMAF-38 were from Andhra Pradesh.

Using 20 ISSR primers to screen for their ability to elucidate polymorphic DNA bands, 131 unambiguous and reproducible amplification products were scored. Of the 131 amplified bands, 109 were polymorphic with an average of 5.45 polymorphic fragments per primer (Table 4). A phenetic UPGMA tree was inferred using dice similarity based on the coefficient dendrogram of ISSR data (Fig. 7). The 60 *A. flavus* isolates show an ancestral bifurcation into two lineages, with isolate UOMMAF-53 being the only representative in one of those lineages (noted as II in Fig. 7), and scoring a similarity coefficient of 0.49. Lineage I included the remaining isolates for which the similarity coefficient was 0.96 despite the high number of bifurcating branches observed (Fig. 7). In the phylogenetic NJ consensus tree (Fig. 8) four clades were supported by a bootsrap value higher than 50%. Three (UOMMAF-54, UOMMAF-55 and UOMMAF-71) of the four isolates of one clade were from Rajasthan, while three (UOMMAF-33, UOMMAF-37 and UOMMAF-49) of the nine isolates of the second clade were from Andhra Pradesh and two (UOMMAF-72 and UOMMAF-74) from Uttarakhand.

In the combined ISSR-RAPD NJ consensus tree nine clades were supported by bootstrap values higher than

Table 6 Accession numbers, phylogenetic data, and aflatoxin profiles for the *A. flavus* sample population

Isolate	Accession number	ITS Clade	RAPD lineage	ISSR lineage	HPTLC[a]	icELISA[b]	mPCR[c]	Molecular identification
Karnataka								
UOMMAF-1	KC480445	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-2	KC480446	B	I	I	–/–/–/–	–	+/+/+/–/+	*A. flavus*
UOMMAF-3	KC480447	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-4	KC480448	Ab	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-5	KC480449	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-6	KC480450	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-7	KC480451	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-8	KC480452	Ab	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-9	KC480453	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-11	KC480455	A	I	I	–/–/–/–	–	+/–/+/+/–	*A. flavus*
UOMMAF-13	KC480457	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-14	KC480458	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-15	KC480459	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-16	KC480460	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-18	KC480462	Ab	I	I	+/–/–/–	+	+/+/+/+/+	*A flavus*
UOMMAF-19	KC480463	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-20	KC480464	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-49	KC480492	Ab	I	I	+/–/–/–	+	+/+/+/+/+	*A flavus*
UOMMAF-65	KC480509	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-66	KC480510	B	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-67	KC480511	B	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
Tamil Nadu								
UOMMAF-23	KC480467	A	I	I	–/+/–/–	+	+/+/+/+/+	*A.. flavus*
UOMMAF-24	KC480468	Ab	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-25	KC480469	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-26	KC480470	A	I	I	–/–/–/–	+	+/–/+/–/–	*A flavus*
UOMMAF-27	KC480471	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-28	KC480472	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-29	KC480473	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
Andhra Pradesh								
UOMMAF-32	KC480476	Ab	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-33	KC480478	Ab	I	I	–/–/–/–	–	+/–/+/–/+	*A. flavus*
UOMMAF-37	KC480480	Ab	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-38	KC480482	A	II	I	+/+/–/–	+	+/+/+/+/+	*A flavus*
UOMMAF-43	KC480487	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-44	KC480488	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-50	KC480494	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-52	KC480496	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-57	KC480501	A	I	I	–/–/–/–	–	+/–/+/–/–	*A. flavus*
UOMMAF-68	KC480512	Ab	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-73	KC480517	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
Gujarat								
UOMMAF-30	KC480474	A	I	I	–/–/–/–	–	+/+/+/+/+	*A. flavus*
Madhya Pradesh								
UOMMAF-46	KC480490	A	I	I	–/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-47	KC480491	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
West Bengal								

Table 6 (continued)

Isolate	Accession number	ITS Clade	RAPD lineage	ISSR lineage	HPTLC[a]	icELISA[b]	mPCR[c]	Molecular identification
UOMMAF-48	KC480493	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
Rajasthan								
UOMMAF-53	KC480497	A	I	II	–/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-54	KC480498	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-55	KC480499	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-56	KC480500	A	I	I	–/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-58	KC480502	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-59	KC480503	Ab	I	I	–/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-60	KC480504	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-61	KC480505	Ab	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-69	KC480513	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-70	KC480514	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-71	KC480515	A	I	I	–/–/–/–	+	+/+/+/+/+	*A. flavus*
Manipur								
UOMMAF-62	KC480506	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
Haryana								
UOMMAF-63	KC480507	A	I	I	+/–/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-64	KC480508	A	I	I	–/–/–/–	–	+/–/+/–/+	*A. flavus*
Uttara Khand								
UOMMAF-72	KC480516	Ab	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*
UOMMAF-74	KC480518	A	I	I	+/+/–/–	–	+/+/+/+/+	*A. flavus*
Himachal Pradesh								
UOMMAF-75	KC480519	A	I	I	+/+/–/–	+	+/+/+/+/+	*A. flavus*

[a]Presence (+) or absence (–) of respective aflatoxins according to HPTLC: $B_1/B_2/G_1/G_2$

[b]Presence (+) or absence (–) of aflatoxins according to icELISA

[c]Presence (+) or absence (–) of respective aflatoxin cluster genes according to mPCR: *ordA*/*aflR*/*omtA*/*ver1*/*norA*

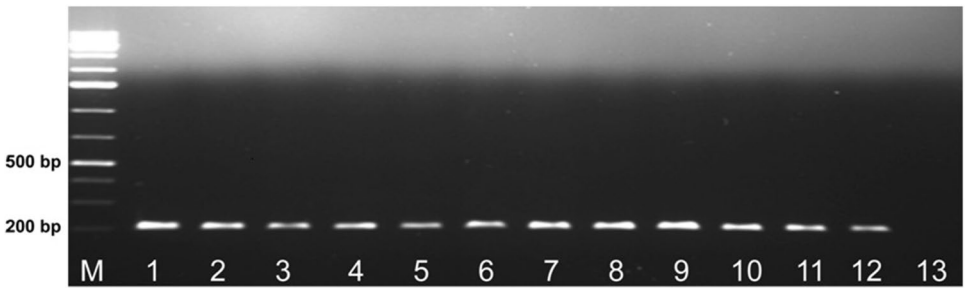

Fig. 2 Amplification products obtained using genera-specific Aspergillopepsin primers for *A. flavus* isolates. Lane M represents marker DNA; lanes 2–12 correspond to UOMMAF isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and lane 13 correspond to negative template control

50% (Fig. 9). Three (UOMMAF-32, UOMMAF-33 and UOMMAF-37) of the four isolates of one clade were from Andhra Pradesh, while two (UOMMAF-72 and UOMMAF-74) of the four isolates of the second clade were from Uttarakhand. In three clades both isolates (UOMMAF-2 and UOMMAF13; UOMMAF-11 and UOMMAF-18; UOMMAF-43 and UOMMAF-54) were from Karnataka.

Discussion

Aflatoxin producing fungi are native to warm arid, semi-arid and tropical regions, but changes in climate may result in greater prevalence of AF contamination outbreaks (Shearer et al. 1992; Bock et al. 2004). Types of aflatoxins and species of AF-producing fungi may differ

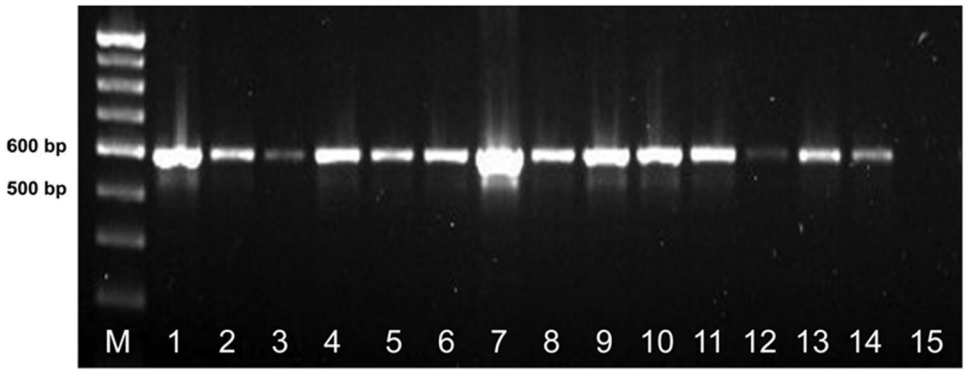

Fig. 3 Amplification products obtained using ITS primers for *A. flavus* isolates. Lane M represents marker DNA; lanes 2–12 correspond to UOMMAF isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and lane 13 correspond to negative template control, respectively

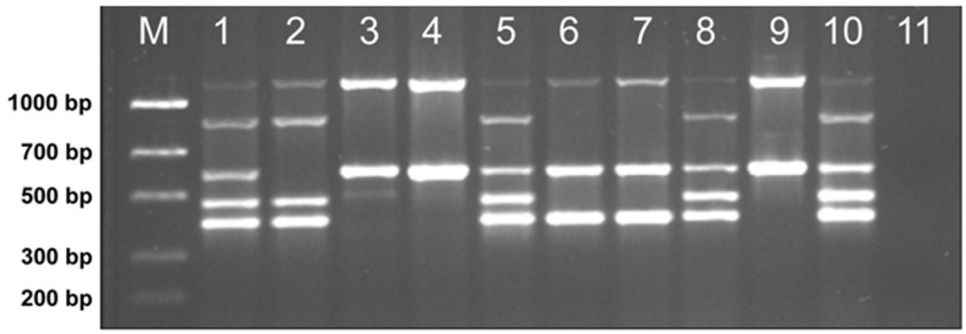

Fig. 4 Amplification products obtained by pentaplex PCR for aflatoxigenic and non-aflatoxigenic *A. flavus* isolates. Lane M represents marker DNA; lanes 2–14 correspond to UOMMAF isolates 1, 2, 11, 26, 30, 33, 64, 71, 57, 75, and 15 correspond to negative template control, respectively. Lanes 2–9 are non-aflatoxigenic isolates

based on geography and climatic conditions (Lisker et al. 1993; Horn and Dorner 1998). In the present work, maize seeds were collected from various agro-climatic regions in India. *Aspergillus* section *Flavi* consists of thirteen species of *Aspergillus* which have few morphological identification features which makes them difficult to identify (Divakara et al. 2014). Among the seed samples collected from Rajasthan and Andhra Pradesh state showed higher *Aspergillus* and aflatoxin contamination compared to other regions. According to previous reports, in maize *A. flavus* infection and AF contamination is prevalent and potentially severe during times of drought (Cotty and Jaime-Garcia 2007).

Aspergillus flavus is widely studied because of AF production, and its mode of action is well-established (Williams et al. 2004). This fungus may also contribute to a wide variety of diseases in plant systems (Amaike and Keller 2011). Unfortunately, no originated efforts have been made to address this problem in India. Scant reports are available in plant systems linked with *Aspergillus* infections. Interaction studies between *A. flavus* and maize seeds revealed that this fungus preferentially colonizes the embryo tissue upon infection, because of its rich nutrient content, and the endosperm is affected late in the destruction of the kernel (Watson 1987; Brown et al. 1994; Keller et al. 1994; Woloshuk et al. 1996). Aflatoxins are reported to be involved in host pathogenesis by affecting amylase activity during embryonic development (Chatterjee 1988).

Toxin analyses revealed that most (52-53/60) of the *A. flavus* isolates in this study are aflatoxigenic, but their aflatoxin levels were not uniform. No aflatoxin G_1 or G_2 production was found in the isolates. Among the aflatoxigenic strains, production varied from 10 to 320 ppb (Supplementary Table). Similar findings were reported from earlier study that variation in AF content is linked with differential expression of the AF biosynthesis genes among *A. flavus* isolates (Gendloff et al. 1992). In nature, strains of *Aspergillus* spp. may lose their ability to produce aflatoxins because of mutations in, or deletions of, biosynthesis pathway genes. Among the 60 isolates tested, eight isolates were found to be non-aflatoxigenic when analyzed by HPTLC; and of those, seven isolates was confirmed as non-aflatoxigenic based on icELISA methods. Additional support was uncovered by different patterns of deletion for AF biosynthesis genes through mPCR analysis. We observed evidence of gene deletion for most of the non-aflatoxigenic isolates, but in the cases of UOMMAF-30 and UOMMAF-71 all the tested genes amplified. This observation suggests that their non-aflatoxigenic phenotype is likely due to either a lack of other AF cluster genes not examined in this study, or the presence of mutations that affect the productivity of one or more AF cluster genes. Similar observations were reported whereby they observed large deletions of the AF gene cluster in atoxigenic *A. flavus* isolates (Chang et al. 2005). Eight distinct deletion patterns were reported, including one in which entire AF gene cluster was absent. Recently, several efforts have

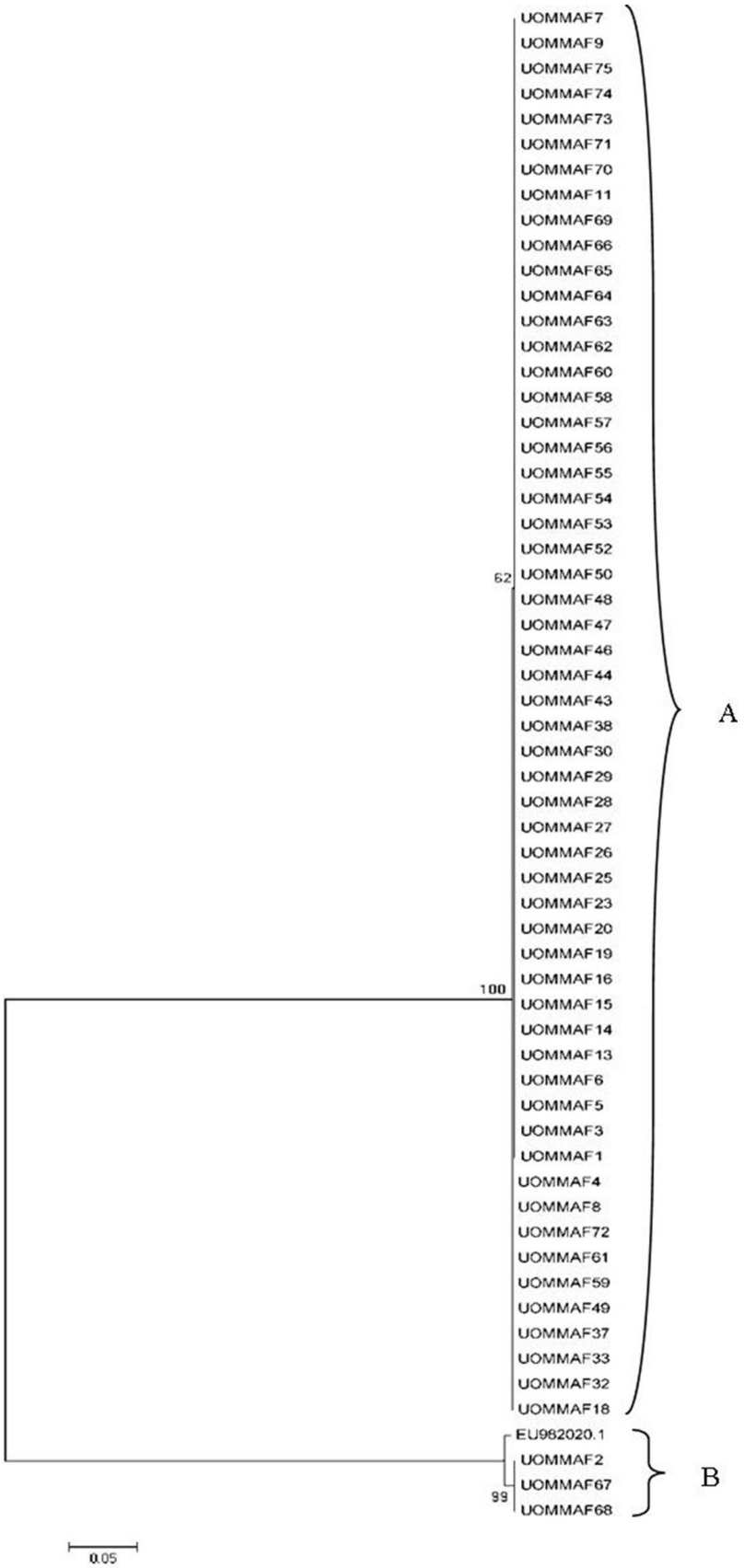

Fig. 5 Phylogenetic tree inferred using ITS sequences from 60 *Aspergillus* sp. isolates sampled across different geographical locations in India. An *A. parasiticus* strain (EU982020.1) was used as the out-group taxa

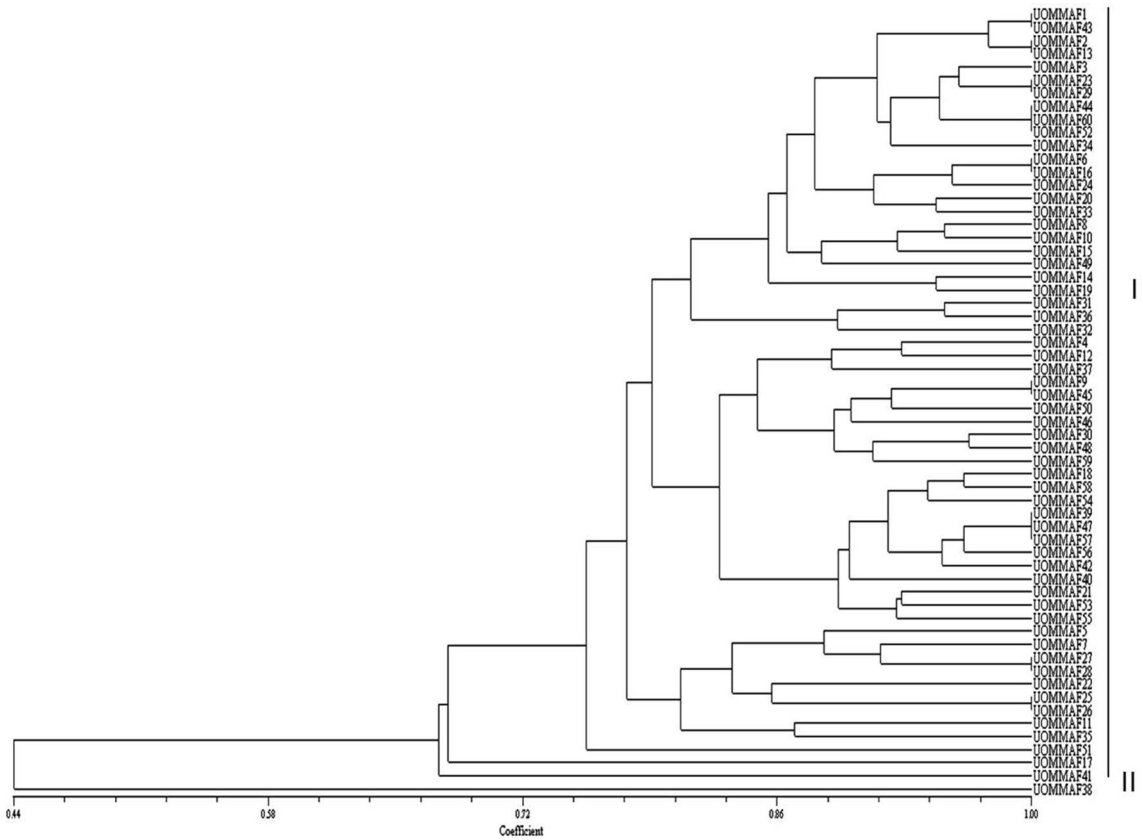

Fig. 6 An inferred UPGMA dendrogram based on DICE similarity coefficient estimates from the RAPD marker analysis of 60 different isolates of *Aspergillus* spp.

been made to distinguish aflatoxigenic and non-aflatoxigenic strains by using mPCR (Giovannetti et al. 2013; Navya et al. 2013; Rashmi et al. 2013). The morphological, physiological and biochemical characterization of the *Aspergillus* isolates failed to group them based on the chemotypic or taxonomic profiles. Optimum aflatoxin production by *A. flavus* occurs at a temperature of 25–30 °C and 0.99 aw conditions, and optimum growth occurs at 30 °C and 0.95 aw in the culture medium (Sanchis and Magan 2004). The enzyme α-amylase has a role in the production of aflatoxins by *A. flavus*, so a deficient α-amylase gene will produce lower quantities of the aflatoxins.

Ecological factors and selection pressures in the distinct agro-climatic regions have the potential to influence genetic diversity (Giovannetti et al. 2013). These influences may lead to variation in the ITS regions among *Aspergillus* species (Henry et al. 2000). The ITS region is often considered a good delineator of species (Schoch et al. 2012). Our ITS analysis revealed little sequence diversity among the 57 isolates which were from lineage I (subgroups Ia and IB) in Clade A. These isolates grouped separately, and all belonged to lineage II the clade B contained UOMMAF-2 (a non-aflatoxigenic isolate), UOMMAF-67, UOMMAF-68 and an *A. parasiticus* isolate from Greece. The presence of the two lineages in *A. flavus* is well known which well reported (Moore et al. 2009). It may be that lineage B isolates actually belong to *A. parasiticus*, because their ITS sequences are very similar to the ITS sequence (EU982020.1) of the isolate, which was identified as *A. parasiticus* by Krimitzas et al. (2013). To show more intra-species genetic diversity among the 60 *A. flavus* isolates may require using additional molecular markers, such as the intergenic regions of the AF gene cluster (Cary et al. 2005).

The 15 RAPD and 20 ISSR primers used in this study provided results suggesting considerable genetic diversity for a majority of *Aspergillus* isolates from 13 different agro-climatic regions of India. The RAPD and ISSR dendrogram exhibited extensive pattern diversity among the majority of the isolates, despite their similarity coefficients of 1.0 and 0.94. Our findings revealed that these *Aspergillus* isolates, which were randomly distributed across India, cannot be grouped based on geography. Furthermore, RAPD and ISSR patterns obtained with these primers failed to discriminate between aflatoxigenic and non-aflatoxigenic phenotypes and between ITS clades A and B. In the present study some isolates from the same region were phylogenetically related to

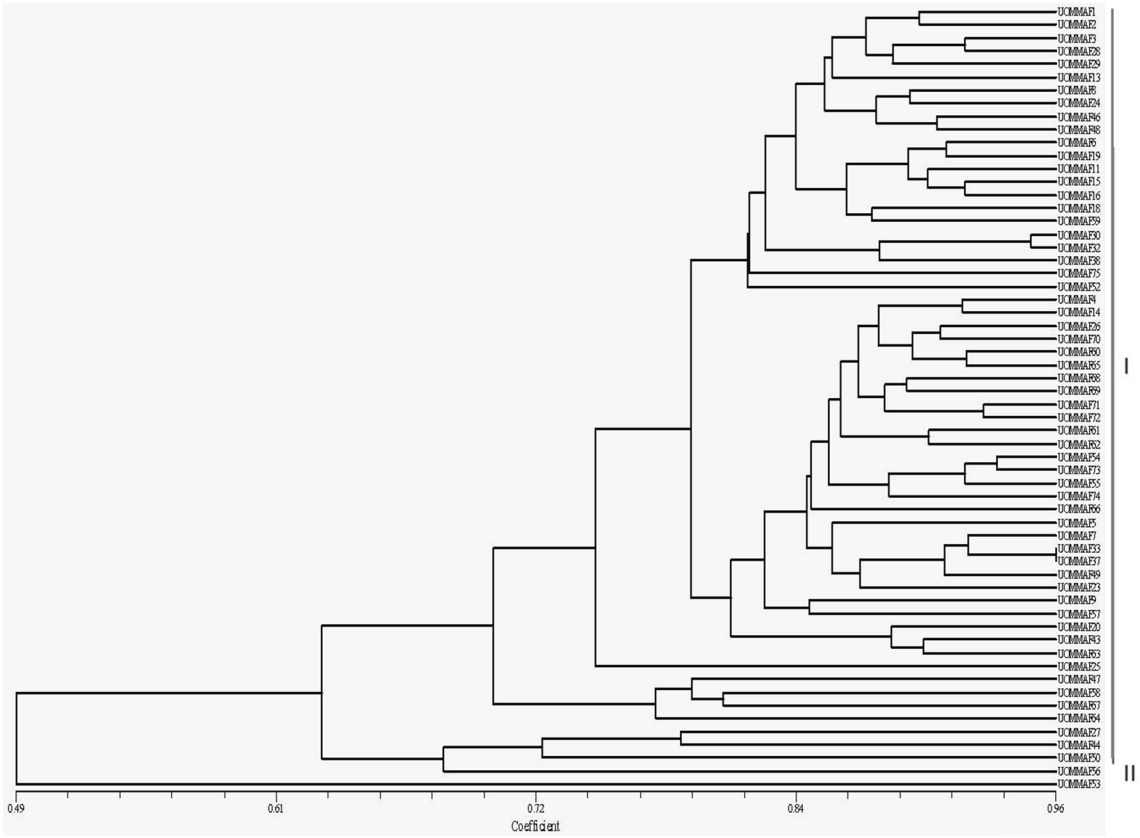

Fig. 7 An inferred UPGMA dendrogram based on DICE similarity coefficient estimates from the ISSR marker analysis of 60 different isolates of *A. flavus*

each other based on the phylogenetic trees of ISSR, RAPD and combined ISSR-RAPD data. Its reported that calmodulin gene and ISSR patterns for investigating intra-species diversity among 30 *A. flavus* isolates from peanut in China, failed to separate isolates based on their toxigenic profiles or their regions of the origin (Zhang et al. 2013).

Conclusions

The present study has provided relevant information on the current status of *A. flavus* infection across different maize-growing regions of India. We show that diverse populations of aflatoxigenic *A. flavus* capable of producing varied levels of aflatoxins, are potential threats for agricultural production. Likewise, from the results of RAPD and ISSR it was concluded that distribution of two lineages of *A. flavus* across India is random, and these strains cannot be grouped based on their agro-climatic regions or based on aflatoxigenicity. Future studies involving a more holistic approach should be conducted to better understand the distribution and diversity of *A. flavus* and aflatoxin contamination in agricultural crops. This will aid in developing suitable strategies for the management of *A. flavus* and aflatoxins in maize.

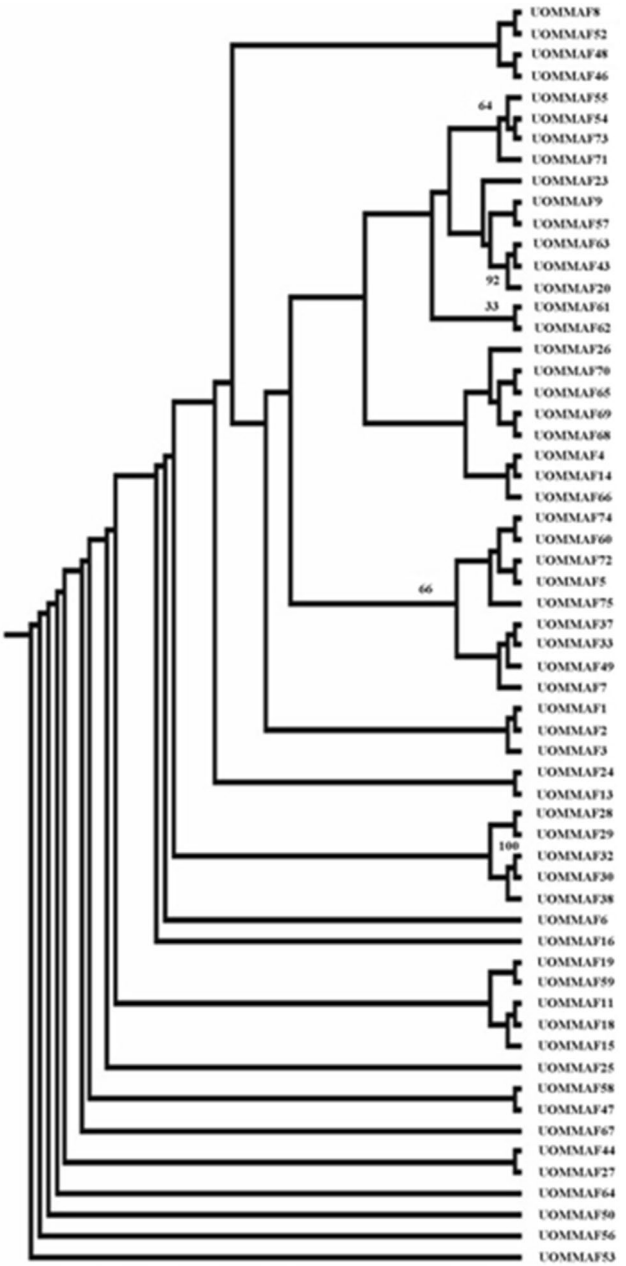

Fig. 8 The neighbor joining tree for ISSR data with bootstrap values generated using PHYLIP Program for 60 different isolates of *A. flavus*

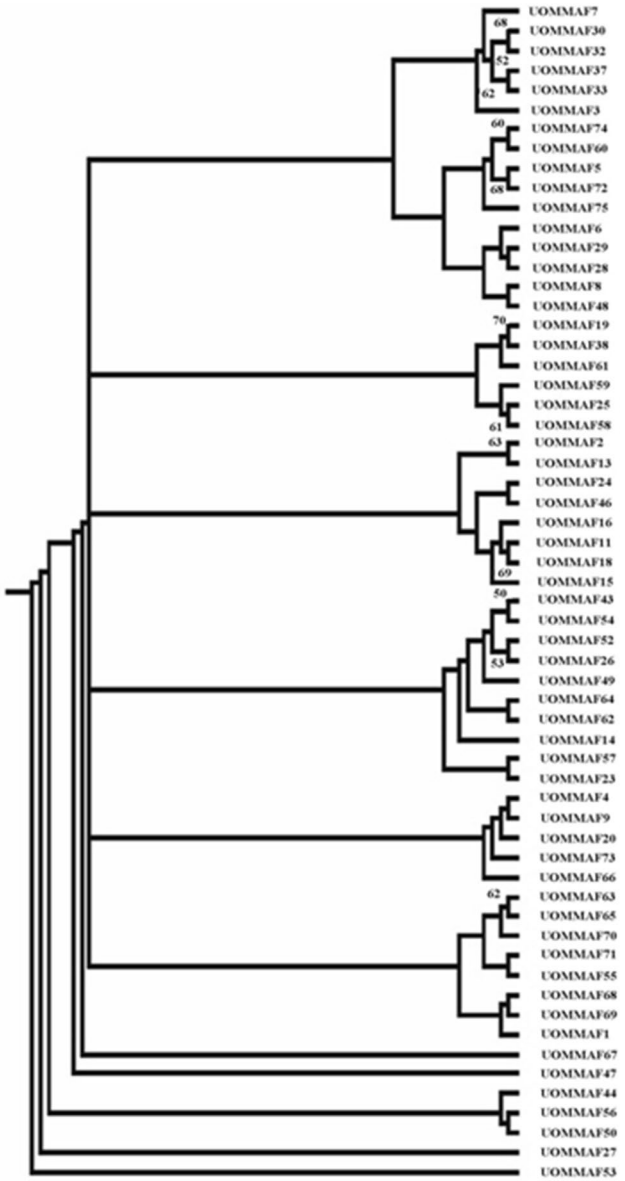

Fig. 9 Combined ISSR-RAPD NJ consensus tree with bootstrap values generated using PHYLIP Program for 60 different isolates of *A. flavus*

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Availability of data and supporting materials section Please contact author for data requests.

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