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Survey of Vietnamese Peanuts, Corn and Soil for the Presence of *Aspergillus flavus* and *Aspergillus parasiticus*

N. Tran-Dinh · I. Kennedy · T. Bui · D. Carter

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Abstract *Aspergillus flavus* and *Aspergillus parasiticus* cause perennial infection of agriculturally important crops in tropical and subtropical areas. Invasion of crops by these fungi may result in contamination of food and feed by potent carcinogenic aflatoxins. Consumption of aflatoxin contaminated foods is a recognised risk factor for human hepatocellular carcinoma (HCC) and may contribute to the high incidence of HCC in Southeast Asia. This study conducted a survey of Vietnamese crops (peanuts and corn) and soil for the presence of aflatoxigenic fungi and used microsatellite markers to investigate the genetic diversity of Vietnamese *Aspergillus* strains. From a total of 85 samples comprising peanut (25), corn (45) and soil (15), 106 strains were isolated. Identification of strains by colony morphology and aflatoxin production found all Vietnamese strains to be *A. flavus* with no *A. parasiticus* isolated. *A. flavus* was present in 36.0% of peanut samples, 31.1% of corn samples, 27.3% of farmed soil samples and was not found in virgin soil samples. Twenty-five per cent of the strains produced aflatoxins. Microsatellite analysis revealed a high level of genetic diversity in the Vietnamese *A. flavus* population. Clustering, based on microsatellite genotype, was unrelated to aflatoxin production, geographic origin or substrate origin.

Keywords Peanuts · Corn · Soil · Microsatellite markers · Genetic diversity

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Introduction

Peanuts and corn are grown extensively in Vietnam and are major agricultural commodities, with 4.6 million metric tons of corn and 0.46 million metric tons of peanuts produced annually [1]. The corn is almost exclusively used as animal feed, while peanuts are consumed by humans and used in the production of vegetable oils. The potential for these crops to be infected by *Aspergillus flavus* and *Aspergillus parasiticus* before or after harvest is a well-recognised problem [2]. One of the main reservoirs of inocula for these fungi is agricultural soil [3]. During periods of drought stress, aflatoxigenic fungi may become the dominant species in soil, due to their ability to grow at high temperatures and at low water activities [4]. Infection of crops is a potential health threat because of the ability of some isolates to produce potent carcinogenic aflatoxins [2].

Exposure to aflatoxin-contaminated food is considered a major risk factor for human hepatocellular carcinoma (HCC) [5–8]. The highest rates of HCC incidence are found in East and Southeast Asia and sub-Saharan Africa [9, 10], and in these developing regions, there is an increasing demand for monitoring of aflatoxins using techniques such as ELISA [11]. The prevalence of HCC in these areas may be due, in part, to a combination of climate and lower standards of farm practices, drying methods and storage conditions, leading to higher levels of aflatoxin in food and feed.

Vietnam lies entirely in the tropics and subtropics, which are climatic areas known to be favourable for the growth of *Aspergillus*, among other fungi. Aflatoxin is a recognised problem in Vietnam, and reducing contamination currently relies on postharvest strategies that prevent excessive fungal growth in food commodities. These can be difficult to implement in very humid areas, however, as seed that is initially dry can develop a water content that is conducive to fungal growth [12]. A promising alternative is to use competitive biological control by colonising soils with nontoxigenic *A. flavus* or *A. parasiticus* strains, which exclude their toxigenic counterparts [13, 14]. However, before biological control strategies can be implemented, an understanding of the occurrence and population diversity of *A. flavus* and *A. parasiticus* in Vietnam is required. Several studies of aflatoxigenic fungi and aflatoxin production have been carried out in Southeast Asia [15–19], but no major survey of Vietnam has been done. The aims of this study were therefore to (1) survey for the presence of *A. flavus* and *A. parasiticus* in Vietnamese crops potentially at risk of aflatoxin contamination, namely peanuts and corn, along with accompanying crop soils and virgin soils; (2) assess whether isolated strains could produce aflatoxins; and (3) investigate their genetic diversity using microsatellite marker.

Materials and Methods

Sampling of Peanuts, Corn and Soil

The field survey of Vietnamese peanuts, corn and soil was conducted in the northern hemisphere winter from 27 February to 19 March 2000. For the purposes of sampling, Vietnam was divided into the Northern region, where the weather was cool (~15–20°C) and wet, and the Southern region where it was hot (~25–30°C) and humid. The Northern regions included the Northern Uplands, the Red River Delta and the Northern Central region (Fig. 1). The Southern regions included the Central Highlands, the South East region and the Mekong Delta (Fig. 1). In the North, samples were collected from the following provinces: Lao Cai, Lang Son, Quang Ninh, Ninh Binh, Son La, Vinh Phu, Ha Bac, Ha Tay, Hoa Binh, Thanh Hoa, Nghe An and Thua Thien. In the South, samples were collected from Dac Lac Province, Dong Nai Province, Tay Ninh Province, Vinh Long Province, Soc Trang Province, Can Tho Province and the surrounding areas of Ho Chi Minh City. The central regions of Vietnam were not extensively surveyed due to recent flooding in the area.

Peanuts and corn were collected from markets, grain depots, farms or homes and had been harvested during the previous growing season. In each case, the supplier was asked from which region they had sourced the crop. Only uncooked peanuts were sampled, while dried and fresh corn was sampled.

Soil samples were collected by first brushing away the top 2 cm of soil and taking a small sample (~100 g) from the next 4–6 cm. Soil samples were taken from farmed and unfarmed or virgin areas. In crop fields, soil samples were collected within 15 cm of a plant at random locations within the field. At the time of collection, immature crop plants were seen growing.

All samples were stored in plastic freezer zip-lock bags. Soil samples that contained high levels of moisture were placed in paper bags and allowed to dry. Samples were kept cool and were refrigerated immediately on arrival at the laboratory. Prior to being brought into Australia at the end of the survey, all samples were stored at −20°C for at least 48 h to kill insects.

Isolation of *A. flavus* and *A. parasiticus*

Peanut, corn and soil samples were mixed thoroughly before being examined for the presence of *A. flavus* and *A. parasiticus* using standard techniques [4]. Peanuts and corn kernels were surface disinfected in 10% household chlorine bleach (i.e. ~0.5% active chlorine) for 2 min, then rinsed twice with water. Twenty kernels from each peanut and corn

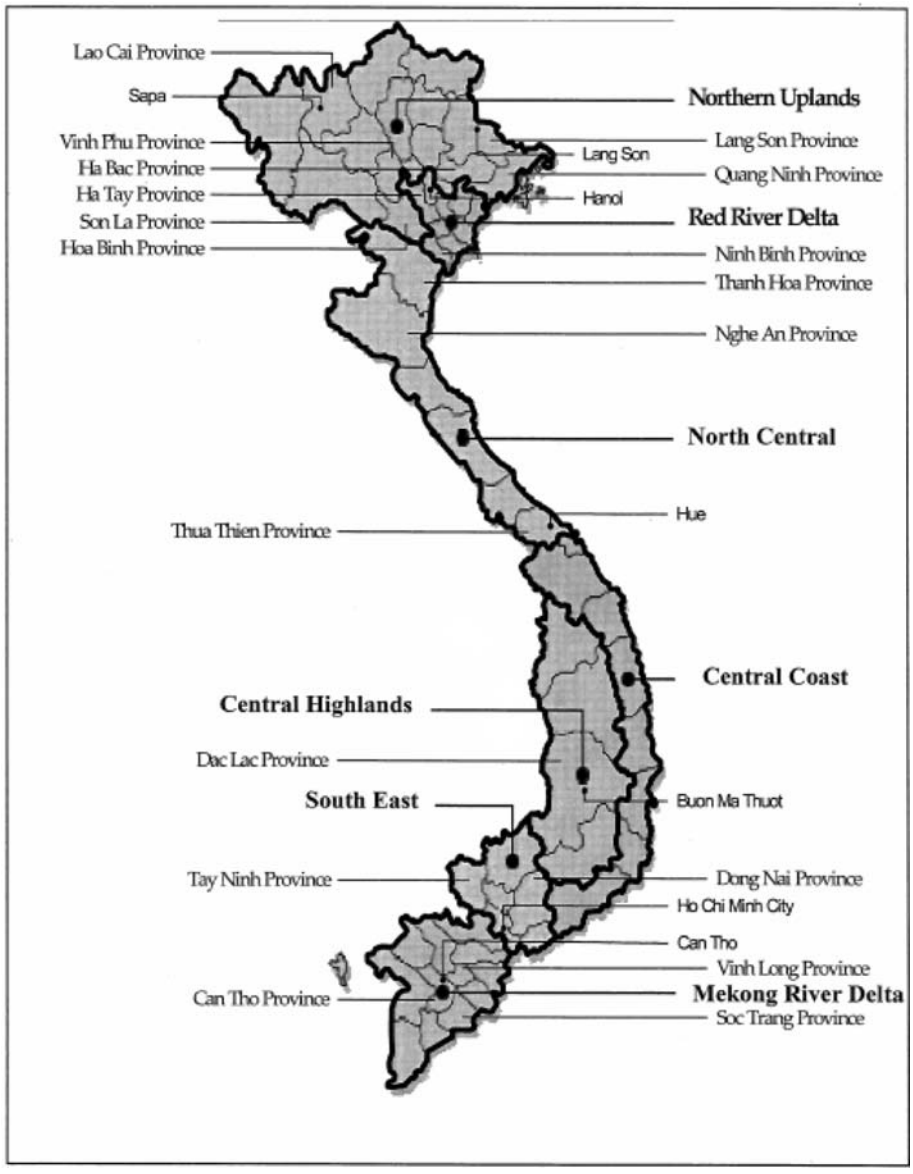


Fig. 1 Map of Vietnam indicating the provinces from which peanuts, corn and soil samples were collected between February and March 2000

sample were randomly selected and transferred onto two *Aspergillus flavus* and *parasiticus* agar (AFPA: 1% peptone, 2% yeast extract, 0.05% ferric ammonium citrate, 0.01% chloramphenicol, 9.7 μM dichloran, 1.5% agar) [20] plates (ten per plate) using sterile forceps. Plates were incubated at 30°C for 3 days.

Soil samples were examined using standard dilution plating techniques onto AFPA plates [20]. Soil samples were mixed thoroughly prior to use. 10 g of soil was added to 0.1% peptone water, mixed vigorously for 30 s and serially diluted to 10^{-5}. 100 μl of each dilution was spread onto two replica AFPA plates. The plates were incubated at 30°C for 3 days. Isolates of *A. flavus* or *A. parasiticus* were recognised by bright orange colouration of the reverse colonies and were subcultured onto new AFPA plates for verification.

Identification of *A. flavus* and *A. parasiticus* Strains

Strains were identified following subculturing on Czapec Yeast Agar (CYA: 0.1% K_2HPO_4, 3% sucrose, 0.5% yeast extract, 0.3% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$, 0.005% $CuSO_4{\cdot}5H_2O$, 0.01% $ZnSO_4{\cdot}7H_2O$, 1.5% agar) media and incubation at 25°C for 7 days [21]. Strains were initially identified macroscopically and confirmed microscopically by conidiophore structure and conidial roughening.

Detection of Aflatoxin Production

Toxin production was assessed by growing strains on coconut cream agar (CCA: 50% coconut cream and 1.5% agar) for 3 days at 30°C and observing colonies under long wavelength (365 nm) ultraviolet light. The appearance of intense fluorescence around fungal colonies was presumptive evidence that a strain could produce aflatoxin. Blue/violet fluorescence indicated that a strain was able to produce B aflatoxin only, while a blue/white fluorescence indicated that a strain produced both B and G aflatoxins [22].

Statistical Analysis of Isolation and Aflatoxin Production Data

Fisher's exact test and the chi-square test were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Microsatellite Marker Amplification and Analysis

Genomic DNA was prepared from Vietnamese strains as described in Tran-Dinh et al. [23].

The microsatellite markers AFPM1-7 were used for analysis of genetic relatedness of Vietnamese strains. Microsatellite amplifications were carried out as described in Tran-Dinh and Carter [24].

Pairwise population distances were calculated from microsatellite allele data using the MICROSAT program, version 1.4 [25]. Null alleles were scored as missing data. The proportional shared allele distance measure (Dps) was used in the MICROSAT program. Pairwise distances were used to construct a dendrograms using the Neighbour-Joining algorithm [26] available in the program PHYLIP 3.5c. Bootstrap analyses were performed using MICROSAT with 1,000 replications and pairwise distance analyses, and construction of consensus trees was performed using the program PHYLIP 3.5c. *A. parasiticus* strain FRR4471, obtained from the Food Science Australia, CSIRO culture collection was used as an out-group for bootstrap analysis. Bootstrap analysis was performed on a randomly selected subset of isolates to minimise computational time.

For determining the probability of a genotype occurring more than once in the dataset, isolates taken from the same sample or region that shared a genotype were removed from the dataset, assuming that these isolates were identical. The probability was then calculated as

$$\sum_{x=n}^{G} \frac{G!}{x!(G-x)!}(P)^x(1-P)^{G-x}$$

where G is the number of genotyped isolates within the population, P is the probability of observation of the original genotype (which is the product of the frequency of each allele found at a locus) and n is the number of isolates with the same genotype as that in question. In this study, $n = 1$ and the formula reduces to $Pse = 1 - (1 - P)^G$ [27].

Results

Isolation of *A. flavus* and *A. parasiticus* Strains and Analysis of Aflatoxin Production

The presence of *A. flavus* and *A. parasiticus* and the potential for aflatoxin production were tested in a total of 85 samples, comprising peanuts (25), corn (45) and soil (15). All *Aspergillus* strains subcultured from AFPA plates onto CYA plates produced yellow–green conidia. Microscopic examination found these to be globose with smooth to finely roughened walls, indicating that all strains were *A. flavus*. This was confirmed by aflatoxin analysis on CCA plates. All Vietnamese strains produced either blue/violet fluorescence or no fluorescence. This indicated that the aflatoxigenic Vietnamese strains produced only B aflatoxins, which is characteristic of *A. flavus*.

A total of 106 strains of *A. flavus* were isolated, and each strain was assigned an identifying number

(Table 1). The results of isolation and aflatoxin production analyses are summarised in Tables 2 and 3. No strains of *A. parasiticus* were found. There was no significant difference among the percentage of peanut, corn or soil samples that were positive for the presence of *A. flavus* ($P = 0.3753$). Likewise, there was no significant difference between the percentage of positive samples from Northern Vietnam (17/47 samples positive) and those from Southern Vietnam (9/38 samples positive) ($P = 0.2388$). When individual crops from the two regions were compared, a slightly significant difference ($P = 0.0441$) in the percentage of positive corn samples was found, with 12/28 and 2/17 positive samples in the North and South, respectively.

CCA analysis found 25.5% of Vietnamese strains produced aflatoxin (Table 3). The percentages of toxigenic strains from peanuts (37.9%), corn (20%) and farmed soil samples (28.6%) were not significantly different ($P = 0.1728$). However, the percentage of toxigenic strains isolated from peanut samples in the North (7.7%) was significantly lower than those isolated from the South (62.5%) ($P = 0.0057$).

Genetic Diversity of Vietnamese Strains

A random selection of 84 strains (Table 1), including 61 from the Northern regions of Vietnam and 23 from the Southern regions, was chosen for analysis of genetic relatedness using the microsatellite markers AFPM1-7. Each isolate was scored for the seven microsatellite markers to produce an overall multilocus genotype for each isolate. The majority of strains, including some isolated from a single sample, had unique multilocus genotypes. Four overall genotypes were shared by two or more strains: 2022, 2024 and 2025; 2056, 2060, 2061, 2062 and 2063; 2078 and 2083; and 2067 and 2090. Strains 2022, 2024 and 2025 were all isolated from the same peanut sample and were considered to be clones. Likewise, strains 2056, 2060, 2061, 2062 and 2063 were all isolated from the same corn sample and were considered to be clones. Strains 2078 and 2083 were both isolated from local corn varieties, but were obtained from different provinces, and strains 2067 and 2090 were both isolated from hybrid corn samples, but were obtained from different provinces. Probability analysis of strains 2078 and 2083 indicated that they were truly identical ($Pse < 0.05$) and did not merely share high

Table 1 Strains isolated from Vietnamese crop and soil samples

Strain no.	Location[a]	Substrata[b]	Aflatoxin production
2001[c]	A: Lao Cai (Sapa)	Corn (L)	Nontoxigenic
2002[c]	A: Lao Cai (Sapa)	Corn (L)	Toxigenic
2003[c]	A: Lao Cai (Sapa)	Corn (L)	Nontoxigenic
2004[c]	A: Lao Cai (Sapa)	Corn (L)	Toxigenic
2005[c]	A: Lao Cai (Sapa)	Corn (L)	Toxigenic
2006[c]	A: Lao Cai (Sapa)	Corn (L)	Toxigenic
2007[c]	F: Soc Trang	Corn (U)	Nontoxigenic
2008[c]	F: Soc Trang	Corn (U)	Nontoxigenic
2009[c]	F: Soc Trang	Corn (U)	Nontoxigenic
2010[c]	F: Soc Trang	Corn (U)	Nontoxigenic
2011[c]	F: Soc Trang	Corn (U)	Nontoxigenic
2012[c]	F: Soc Trang	Corn (U)	Nontoxigenic
2013[c]	D: Dac Lac	Corn (U)	Toxigenic
2014[c]	D: Dac Lac	Peanut	Toxigenic
2015[c]	C: Thua Thien	Peanut	Nontoxigenic
2016[c]	C: Thua Thien	Peanut	Toxigenic
2017[c]	D: Dac Lac	Peanut	Toxigenic
2018[c]	D: Dac Lac	Peanut	Toxigenic
2019[c]	D: Dac Lac	Peanut	Toxigenic
2020[c]	D: Dac Lac	Peanut	Nontoxigenic
2021	D: Dac Lac	Peanut	Nontoxigenic
2022[c]	D: Dac Lac	Peanut	Toxigenic
2023[c]	D: Dac Lac	Peanut	Nontoxigenic
2024[c]	D: Dac Lac	Peanut	Toxigenic
2025[c]	D: Dac Lac	Peanut	Toxigenic
2026[c]	D: Dac Lac	Peanut	Toxigenic
2027[c]	D: Dac Lac	Peanut	Nontoxigenic
2028[c]	D: Dac Lac	Peanut	Toxigenic
2029[c]	D: Dac Lac	Peanut	Toxigenic
2030[c]	D: Dac Lac	Peanut	Nontoxigenic
2031[c]	D: Dac Lac	Peanut	Nontoxigenic
2032[c]	B: Ninh Binh	Peanut	Nontoxigenic
2033[c]	B: Ha Tay	Peanut	Nontoxigenic
2034[c]	C: Thanh Hoa	Peanut	Nontoxigenic
2035[c]	C: Thanh Hoa	Peanut	Nontoxigenic
2036[c]	C: Thanh Hoa	Peanut	Nontoxigenic
2037[c]	C: Thanh Hoa	Peanut	Nontoxigenic
2038[c]	C: Thanh Hoa	Peanut	Nontoxigenic
2039[c]	B: Ninh Binh	Corn (H)	Nontoxigenic
2040	C: Thanh Hoa	Corn (H)	Nontoxigenic
2041	C: Thanh Hoa	Corn (H)	Nontoxigenic
2042[c]	C: Thanh Hoa	Corn (H)	Nontoxigenic
2043	C: Thanh Hoa	Corn (H)	Nontoxigenic

Table 1 continued

Strain no.	Location[a]	Substrata[b]	Aflatoxin production
2044[c]	C: Thanh Hoa	Corn (H)	Nontoxigenic
2045[c]	C: Thanh Hoa	Corn (H)	Nontoxigenic
2046[c]	C: Thanh Hoa	Corn (H)	Nontoxigenic
2047[c]	C: Thanh Hoa	Corn (H)	Toxigenic
2048[c]	C: Thanh Hoa	Corn (H)	Nontoxigenic
2049[c]	A: Son La	Corn (H)	Nontoxigenic
2050	A: Son La	Corn (H)	Nontoxigenic
2051[c]	A: Son La	Corn (H)	Nontoxigenic
2052[c]	A: Son La	Corn (H)	Toxigenic
2053[c]	A: Son La	Corn (H)	Nontoxigenic
2054[c]	A: Son La	Corn (H)	Toxigenic
2055[c]	A: Son La	Corn (H)	Nontoxigenic
2056[c]	A: Lang Son	Corn (H)	Nontoxigenic
2057	A: Lang Son	Corn (H)	Nontoxigenic
2058	A: Lang Son	Corn (H)	Nontoxigenic
2059[c]	A: Lang Son	Corn (H)	Nontoxigenic
2060[c]	A: Lang Son	Corn (H)	Nontoxigenic
2061[c]	A: Lang Son	Corn (H)	Nontoxigenic
2062[c]	A: Lang Son	Corn (H)	Nontoxigenic
2063[c]	A: Lang Son	Corn (H)	Nontoxigenic
2064	A: Lang Son	Corn (H)	Nontoxigenic
2065[c]	A: Quang Ninh	Corn (H)	Nontoxigenic
2066[c]	A: Quang Ninh	Corn (H)	Toxigenic
2067[c]	A: Quang Ninh	Corn (H)	Nontoxigenic
2068[c]	A: Quang Ninh	Corn (H)	Nontoxigenic
2069[c]	A: Quang Ninh	Corn (H)	Toxigenic
2070[c]	A: Quang Ninh	Corn (H)	Nontoxigenic
2071	A: Quang Ninh	Corn (H)	Nontoxigenic
2072	A: Quang Ninh	Corn (H)	Toxigenic
2073	A: Quang Ninh	Corn (H)	Nontoxigenic
2074	A: Quang Ninh	Corn (H)	Toxigenic
2075	A: Son La	Corn (H)	Nontoxigenic
2076	A: Son La	Corn (H)	Nontoxigenic
2077	A: Son La	Corn (H)	Nontoxigenic
2078[c]	A: Lao Cai (Sapa)	Corn (L)	Nontoxigenic
2079[c]	A: Hoa Binh	Corn (L)	Nontoxigenic
2080	A: Hoa Binh	Corn (L)	Nontoxigenic
2081[c]	A: Hoa Binh	Corn (L)	Nontoxigenic
2082	A: Hoa Binh	Corn (L)	Nontoxigenic
2083[c]	A: Hoa Binh	Corn (L)	Nontoxigenic
2084[c]	A: Hoa Binh	Corn (L)	Nontoxigenic
2085[c]	A: Lao Cai (Sapa)	Peanut	Nontoxigenic
2086[c]	A: Lao Cai (Sapa)	Peanut	Nontoxigenic
2087[c]	B: Ha Tay	Peanut	Nontoxigenic
2088[c]	B: Ninh Binh	Peanut	Nontoxigenic
2089[c]	A: Lao Cai (Sapa)	Corn (H)	Nontoxigenic
2090[c]	A: Lao Cai (Sapa)	Corn (H)	Nontoxigenic
2091	A: Lao Cai (Sapa)	Corn (H)	Toxigenic
2092	A: Lao Cai (Sapa)	Corn (H)	Toxigenic
2093[c]	A: Lao Cai (Sapa)	Corn (Se)	Toxigenic
2094	A: Lao Cai (Sapa)	Corn (St)	Nontoxigenic
2095[c]	B: Ninh Binh	Corn (H)	Nontoxigenic
2096[c]	B: Ninh Binh	Corn (H)	Nontoxigenic
2097	A: Son La	Corn (H)	Nontoxigenic
2098	A: Son La	Corn (H)	Nontoxigenic
2099[c]	A: Son La	Corn (H)	Nontoxigenic
2100[c]	E: Dong Nai	Soil	Nontoxigenic
2102[c]	E: Dong Nai	Soil	Toxigenic
2103[c]	E: Dong Nai	Soil	Toxigenic
2105[c]	F: Can Tho	Soil	Nontoxigenic
2106[c]	F: Can Tho	Soil	Nontoxigenic
2107[c]	F: Can Tho	Soil	Nontoxigenic
2108[c]	F: Can Tho	Soil	Nontoxigenic

[a] *A* Northern Uplands, *B* Red River Delta, *C* North Central Region, *D* Central Highlands, *E* South East Region, *F* Mekong River Delta

[b] Bracketed information refers to corn variety—*H* hybrid corn, *L* local corn, *Se* seed corn, *St* sticky corn, *U* unknown corn variety

[c] Strain used in genetic diversity study

frequency microsatellite alleles. Similarly, strains 2067 and 2090 were truly identical ($Pse < 0.05$).

Figure 2 shows the genetic relationship between the 84 strains of *A. flavus* isolated from Vietnam. A high level of genetic diversity was seen in the 84 strains with no evident correlation between strain toxigenicity and genotype. No correlation between geographic origin of strains and genotype was evident either. For example, the strains collected from Sapa (Lao Cai Province) in the Northern Uplands, were interspersed throughout the dendrogram and showed no clustering. Unless they were clonally related, strains isolated from a particular sample type generally did not cluster together. Strains isolated from peanut samples, from both Northern and Southern regions, were interspersed throughout the dendrogram. However, 21 strains isolated from hybrid corn samples grouped together. These strains were also all

Table 2 Presence of *A. flavus* in crop and soil samples

Sample type	No. of samples tested	Location								No. (%) of positive samples
		North				South				
		A	B	C	Total	D	E	F	Total	
Peanuts	25	1/5[a]	2/6	2/4	5/15	4/8	0/2	–	4/10	9 (36.0%)
Corn	45	10/21	1/6	1/1	12/28*	1/7	0/2	1/8	2/17*	14 (31.1%)
Soil-farmed	11	0/1	–	–	0/1	0/1	2/6	1/3	3/10	3 (27.3%)
Soil-virgin	4	0/1	0/2	–	0/3	0/1	–	–	0/1	0
Total	85				17/47				9/38	26 (31.0%)

A Northern Uplands, *B* Red River Delta, *C* North Central Region, *D* Central Highlands, *E* South East Region, *F* Mekong River Delta

* Significant difference ($P < 0.05$) was found in the levels of infection in corn between Northern and Southern samples

[a] Number of infected samples/number of samples tested

Table 3 Number of aflatoxigenic strains of *A. flavus*

Sample type	Location								No. (%) of aflatoxin-producing strains
	North				South				
	A	B	C	Total	D	E	F	Total	
Peanuts	0/2[a]	0/4	1/7	1/13* (7.7%)	10/16	–	–	10/16* (62.5%)	11/29 (37.9%)
Corn	11/51	0/3	2/9	13/63 (20.6%)	1/1	–	0/6	1/7 (14.3%)	14/70 (20.0%)
Soil	–	–	–	–	–	2/3	0/4	2/7	2/7 (28.6%)
Total				14/76 (18.4%)				13/30 (43.3%)	27/106 (25.5%)

A Northern Uplands, *B* Red River Delta, *C* North Central Region, *D* Central Highlands, *E* South East Region, *F* Mekong River Delta

* Significant difference in the per cent toxigenic strains isolated from peanuts from Northern and Southern samples ($P < 0.05$)

[a] Number of aflatoxin-producing strains/number of strains isolated

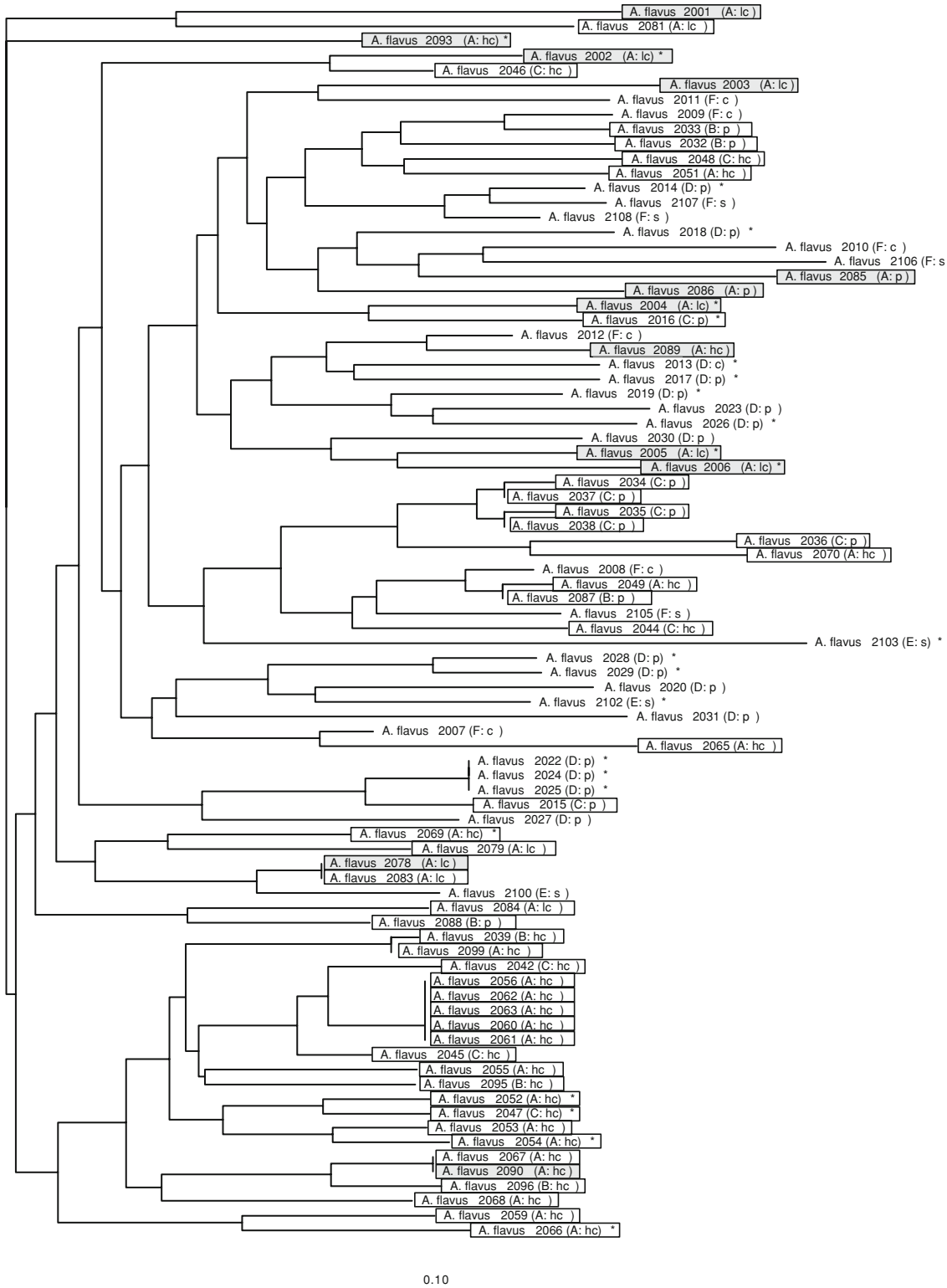
isolated from Northern regions. However, bootstrap analysis of a subset of 34 selected strains (Fig. 3) revealed no support for this clustering.

Discussion

This survey was undertaken as part of a larger project examining mycotoxins in Vietnamese crops [28]. As well as being important in the health and economy of the region, understanding the prevalence and diversity of aflatoxigenic fungi in Vietnam is an important part of our overall understanding of the global structure of these organisms. It was also of interest to investigate fungal contamination of host crops that have been cultivated in remote areas for generations.

All the *Aspergillus* strains isolated from the Vietnamese survey were *A. flavus*, and no *A. parasiticus* strains were recovered. *A. parasiticus* appears to be very uncommon in Southeast Asia, with surveys reporting it to be very rare or absent in Thailand [17, 29] and China [30]. In Japan and the Philippines, however, *A. parasiticus* occurs alongside *A. flavus*, [31, 32], and what determines its exclusion in some regions is not known. This finding is important for the implementation of biocontrol strategies in Vietnam, as only *A. flavus* needs to be considered.

A. flavus was found in 36% of peanut samples, 31.1% of corn samples, 27.3% of farmed soil samples and was not recovered from virgin soil samples. Pitt et al. [17] found the levels of *A. flavus* infection of corn and peanut samples from Thailand to be significantly higher at 85 and 95%, respectively. The proportion of toxigenic strains in Vietnam (25.5%) was also significantly different ($P < 0.05$) to the ~50:50 ratio of toxigenic to nontoxigenic strains reported elsewhere in the world [33–37]. These differences may be due to differences in


A. flavus 2001 (A: lc)
A. flavus 2081 (A: lc)
A. flavus 2093 (A: hc) *
A. flavus 2002 (A: lc) *
A. flavus 2046 (C: hc)
A. flavus 2003 (A: lc)
A. flavus 2011 (F: c)
A. flavus 2009 (F: c)
A. flavus 2033 (B: p)
A. flavus 2032 (B: p)
A. flavus 2048 (C: hc)
A. flavus 2051 (A: hc)
A. flavus 2014 (D: p) *
A. flavus 2107 (F: s)
A. flavus 2108 (F: s)
A. flavus 2018 (D: p) *
A. flavus 2010 (F: c)
A. flavus 2106 (F: s)
A. flavus 2085 (A: p)
A. flavus 2086 (A: p)
A. flavus 2004 (A: lc) *
A. flavus 2016 (C: p) *
A. flavus 2012 (F: c)
A. flavus 2089 (A: hc)
A. flavus 2013 (D: c) *
A. flavus 2017 (D: p) *
A. flavus 2019 (D: p) *
A. flavus 2023 (D: p)
A. flavus 2026 (D: p) *
A. flavus 2030 (D: p)
A. flavus 2005 (A: lc) *
A. flavus 2006 (A: lc) *
A. flavus 2034 (C: p)
A. flavus 2037 (C: p)
A. flavus 2035 (C: p)
A. flavus 2038 (C: p)
A. flavus 2036 (C: p)
A. flavus 2070 (A: hc)
A. flavus 2008 (F: c)
A. flavus 2049 (A: hc)
A. flavus 2087 (B: p)
A. flavus 2105 (F: s)
A. flavus 2044 (C: hc)
A. flavus 2103 (E: s) *
A. flavus 2028 (D: p) *
A. flavus 2029 (D: p) *
A. flavus 2020 (D: p)
A. flavus 2102 (E: s) *
A. flavus 2031 (D: p)
A. flavus 2007 (F: c)
A. flavus 2065 (A: hc)
A. flavus 2022 (D: p) *
A. flavus 2024 (D: p) *
A. flavus 2025 (D: p) *
A. flavus 2015 (C: p)
A. flavus 2027 (D: p)
A. flavus 2069 (A: hc) *
A. flavus 2079 (A: lc)
A. flavus 2078 (A: lc)
A. flavus 2083 (A: lc)
A. flavus 2100 (E: s)
A. flavus 2084 (A: lc)
A. flavus 2088 (B: p)
A. flavus 2039 (B: hc)
A. flavus 2099 (A: hc)
A. flavus 2042 (C: hc)
A. flavus 2056 (A: hc)
A. flavus 2062 (A: hc)
A. flavus 2063 (A: hc)
A. flavus 2060 (A: hc)
A. flavus 2061 (A: hc)
A. flavus 2045 (C: hc)
A. flavus 2055 (A: hc)
A. flavus 2095 (B: hc)
A. flavus 2052 (A: hc) *
A. flavus 2047 (C: hc) *
A. flavus 2053 (A: hc)
A. flavus 2054 (A: hc) *
A. flavus 2067 (A: hc)
A. flavus 2090 (A: hc)
A. flavus 2096 (B: hc)
A. flavus 2068 (A: hc)
A. flavus 2059 (A: hc)
A. flavus 2066 (A: hc) *
0.10

◄ **Fig. 2** Dendrogram showing genetic relatedness of 84 strains of *A. flavus* from Vietnam. Strains from Northern regions are *boxed*. Information appearing in *parentheses* refers to specific region of geographic origin and the sample type the strain was isolated from. *A*, Northern Uplands; *B*, Red River Delta; *C*, North Central Region; *D*, Central Highlands; *E*, South East Region; *F*, Mekong River Delta; *p*, peanut sample; *s*, soil sample; *hc*, hybrid corn variety sample; *lc*, local corn variety sample; *c*, unknown corn variety sample. * Indicates toxigenic strains, nontoxigenic strains are *unmarked*. *Shaded strains* are from Sapa (Lao Cai Province)

prevailing climatic conditions in the regions analysed, the cultivars grown, local agricultural practices and the incidence of insect damage [38–40]. Seasonal variations in these factors also affect the severity of infection [12, 41]. Shearer et al. [41] in a survey of corn and soil in Iowa, USA, found the amount of aflatoxin-producing strains could vary year to year from 15 to 65%. Further sampling will help assess

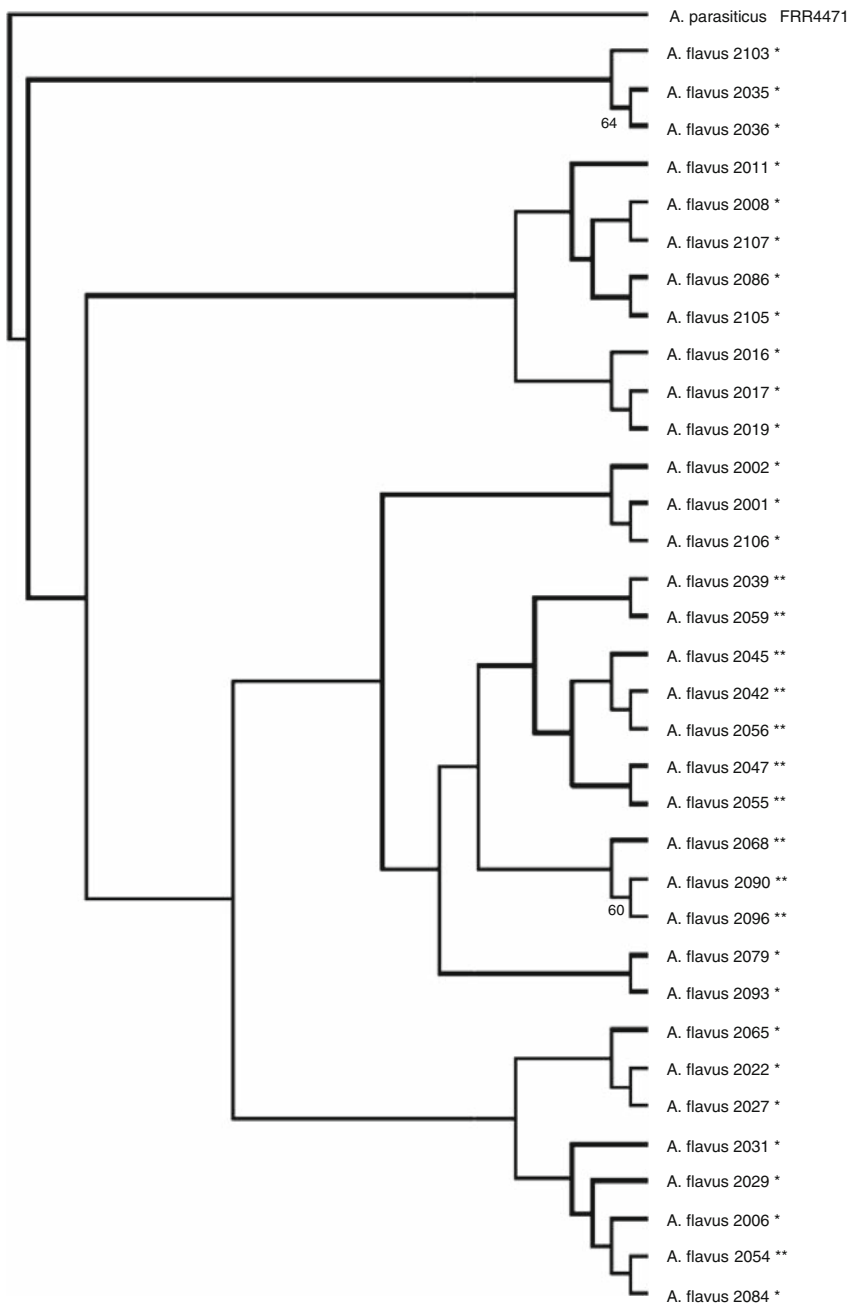


Fig. 3 Cladogram showing relationship of 34 representative *A. flavus* strains. Strains labelled with the same number of *asterisks* clustered together in Fig. 2. Numbers at branch nodes represent bootstrap percentages of 1,000 replications. Only bootstrap values greater than 50 are shown. *A. parasiticus* FRR4471 was used as an outgroup for this analysis

how vulnerable Vietnamese crops are to aflatoxin contamination throughout seasonal and annual fluctuations.

Microsatellite analysis of the *A. flavus* strains revealed a high level of genetic diversity. In many instances, multiple *A. flavus* strains with different genotypes were found to be infecting the same crop sample or soil sample, which is consistent with other studies [42, 43]. High genetic diversity can be indicative of a population that has been present in a region over sufficient evolutionary time to acquire variation or it may be due to a single introduction of highly diverse strains or multiple, independent introductions. High genetic diversity can also be due to sexual recombination. *A. flavus* is thought to be asexual, but population genetic analysis has found evidence of recombination among isolates [44]. A sexual cycle was recently discovered in *Aspergillus fumigatus* [45], and it is probable that sex occurs but is yet to be seen in other *Aspergillus* species.

No correlation was found between microsatellite genotype and the ability to produce aflatoxin, which is consistent with other studies that found aflatoxigenicity to be polyphyletic [23]. There was likewise no correlation between genotype and geographic origin or the sample substrate. It was thought that strains collected from the geographically remote area of Sapa, where local corn varieties are grown and little or no overseas hybrid corn have been planted, might show genetic differentiation. However, the Sapa strains were found interspersed throughout the dendrogram of Vietnamese strains. A group consisting of strains isolated from imported hybrid corn from the Northern regions of Vietnam was seen in Fig. 2, but this cluster was not supported by bootstrap analysis (Fig. 3). Overall, it appears that the Vietnamese *A. flavus* populations are very diverse, cosmopolitan and genetically connected.

Some strains, isolated from the same sample had only very slight differences in genotype and shared a number of microsatellite alleles. For example, strains 2034, 2035, 2037 and 2038 were all isolated from the same peanut sample and were closely related but different from each other (Fig. 2). This level of genetic differentiation may be due to microevolutionary changes within the microsatellite alleles of individual strains.

Although, in relative terms, the level of infection of Vietnamese crops by aflatoxigenic *A. flavus* strains was low, it is evident that infection is occurring, and aflatoxin contamination is a likely result of this infection. Vietnam may be at particular risk of aflatoxin contamination due to the lack of practice of harvest and postharvest techniques able to prevent mould growth. Agricultural practices can strongly influence aflatoxin contamination [39, 46]. Many of the farms and houses visited during the survey stored peanut or corn crops with little concern for the potential of mould growth. Farmers and householders often allowed stored corn to become visibly mouldy, as it was almost exclusively used for animal feeds. This may lead to loss of productivity and degradation of meat quality in animals [47]. In addition, consumption of meat from animals exposed to aflatoxins may result in secondary mycotoxicoses in humans, as aflatoxin may be present in meat as aflatoxin M, a less potent but nonetheless toxic and carcinogenic derivative of aflatoxin B1 [48].

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