Genotypic Diversity of *Aspergillus parasiticus* in an Illinois Corn Field

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ABSTRACT

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Aspergillus parasiticus was isolated from direct platings of soil from a corn field near Kilbourne, Illinois. Soil contained 0.2 to 4.0 CFU of *Aspergillus flavus* and/or *A. parasiticus* per g of soil. Sixty isolates of *A. parasiticus*, each from a separately collected soil sample, were examined for ability to produce sclerotia and aflatoxins, and were subjected to DNA fingerprinting. *Pst*I digests of total genomic DNA from each isolate were probed using the pAF28 repetitive sequence. Among 60 isolates analyzed, 33 (55%) distinct DNA fingerprint groups were identified (each group sharing less than 80% pAF28 band similarity), including 50 distinct genotypes (83%) with less than 100% pAF28 band similarity. A single *A. parasiticus* fingerprint group represented 13% of the sample population. The 83% genotypic diversity of the *A. parasiticus* population was equivalent to the 81% genotypic diversity recorded earlier for a population of 31 *A. flavus* isolates from the same field soil. Sclerotia were produced by 82% of the 50 *A. parasiticus* genotypes during dark incubation at 25°C. All isolates of *A. parasiticus* produced aflatoxin B₁B₂ and G₁G₂, whereas only 36% of the 31 *A. flavus* isolates from these soils produced aflatoxins.

Additional keywords: cluster analysis, latitude, restriction fragments, *Zea mays*

Aspergillus parasiticus Speare was first identified from agricultural soils within the United States corn belt by Angle et al. (1), who investigated aflatoxin production by *Aspergillus flavus* Link:Fr. group isolates from experimental maize cropping systems at Sanborn Field, University of Missouri, Columbia. Fifty-seven percent of these isolates were determined to be *A. parasiticus* and 43% *A. flavus*. It has been argued that *A. parasiticus* is better adapted to a soil environment than *A. flavus* because it is more prevalent in peanut seeds than in aboveground crops (8). Wicklow et al. (31) demonstrated that *A. parasiticus* conidia and sclerotia can survive soil burial for 3 years in a central Illinois field. In southern

Georgia, the diversity of vegetative compatibility groups (VCGs), expressed as the number of groups divided by the total numbers of isolates, was higher for *A. flavus* (0.56) than for *A. parasiticus* (0.22) (12). The *A. parasiticus* population showed a nonrandom distribution, suggesting to Horn and Green (12) that isolates of the same VCG within a soil sample represent to some degree a single clone, and that the population of *A. parasiticus* is less diverse because conidial dispersal is more restricted than in *A. flavus*.

A. flavus and *A. parasiticus* are known primarily from subtropical and tropical habitats (10,24). Manabe et al. (16) surveyed soils throughout Japan and reported both *A. flavus* and *A. parasiticus* in more southern islands, with the distribution of *A. flavus* extending further north than that of *A. parasiticus*. The discovery of mixed soil populations of *A. flavus* and *A. parasiticus* in the midwestern corn belt (1,26,30) invites contrasts of phenotypic characters and genotypic diversity of clonal populations isolated from cooler latitudes. For example, Wicklow and Cole (29) found that cyclopiazonic acid was more commonly detected in *A. flavus* sclerotia from warmer latitudes (82% occurrence) than from cooler latitudes (26% occurrence). In the present study, we evaluated the genotypic diversity (DNA fingerprinting) of 60 *A. parasiticus* isolates from soil samples collected in 1990 to 1991 from within the 16.2-ha University of Illinois River Valley Sand Field (IRVSF), Kilbourne, Illinois. Each isolate was also examined for its ability to produce sclerotia and aflatoxins.

MATERIALS AND METHODS

Isolation of fungal strains. All *A. parasiticus* strains examined in this study were isolated from soil (Bloomfield sand) sampled from IRVSF, Kilbourne, in 1990 to 1991. Soil (0.5 g per petri dish) was directly plated by suspending 0.5 g of soil in 20 ml of melted M3510B agar medium amended with 10% NaCl and incubating the plates for 5 days at 37°C (26). *A. parasiticus* was of common occurrence in these soil isolation plates. To minimize the chance of isolating strains from a single *A. parasiticus* clone, DNA hybridization analyses were performed on one randomly selected *A. parasiticus* isolate from each petri dish. The total number of filamentous fungi in IRVSF soil samples was estimated by dilution plating onto dextrose peptone yeast extract agar (21) followed by a 6-day incubation at 25°C.

Sclerotium production. Sclerotium production was recorded for single-spored cultures after 21 days of dark incubation at 25°C on slants of Czapek agar. Individual strains that did not produce sclerotia on Czapek agar were cultured on slants of potato dextrose agar containing 0.02% yeast extract for 21 days at 25°C and examined for sclerotia.

Aflatoxin determinations. The ability of *A. parasiticus* strains to produce aflatoxins was determined using a procedure developed at the National Peanut Research Laboratory (14). Individual strains were cultured for 5 days at 28°C in 1.0 ml of broth consisting of 190 g of glucose, 20 g of yeast extract, and 10 g of Bacto-soytone (Difco Laboratories, Detroit, MI) per liter in 2.0-ml vials capped with a Teflon septum (Waters Chromatography Division, Millipore Co., No. 73005). Following incubation, 1 ml of chloroform was added to each vial, and the vials were vortexed 15 to 20 s and left standing for 1 to 2 min for solvent partitioning. The presence of aflatoxins was determined by thin-layer chromatography (2). A microliter syringe was inserted through the Teflon cap, and 20 µl of the chloroform layer was removed and spotted on TLC plates along with aflatoxin B₁, B₂, G₁, G₂ standards.

DNA extraction. Fungal mycelia used for DNA extractions were grown from *A. parasiticus* conidial or cell suspensions (25,000 CFU/ml of culture medium) in 500-ml flasks containing 200 ml of yeast extract-peptone-dextrose (YEPD) broth (3 g of yeast extract, 10 g of peptone, and 20 g of dextrose in 1 liter of distilled water). The flasks were incubated on a rotary

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shaker (200 rpm) for 22 to 24 h at 32°C. Following incubation, mycelia were harvested by filtering through a Büchner funnel with sterile Whatman no. 1 filter paper and rinsed twice with sterile distilled water. The mycelial mat was transferred to a disposable 50-ml Sarstedt tube, frozen at -80°C, and lyophilized for 24 h or until the mycelium was completely brittle and dry. DNA from fungal strains was isolated and purified using a previously reported method (22).

DNA hybridization and fingerprint analyses. Fungal DNA was digested to completion with the restriction endonuclease *Pst*I (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's recommendations. Restriction fragments of total DNA (5 µg of digested DNA per lane) were separated by electrophoresis in a 0.8% agarose gel in TAE buffer (0.04 M Tris-acetate, pH 8.0, 0.001 M EDTA) at 1.5 V/cm for 20 h and visualized by staining with ethidium bromide and illumination with ultraviolet light. Restriction fragments were transferred from agarose gels to nylon membranes (Hybond-N, Amersham International, Buckinghamshire, Eng.) using a vacuum blotter (Model 785, Bio-Rad Laboratories, Melville, NY) according to the manufacturer's protocol. Probes were labeled and DNA fingerprints were observed using the nonradioactive CSPD

detection system and "Genius System" (Boehringer Mannheim). Membranes were exposed to X-ray film (Kodak) at 21 ± 2°C for 15 to 30 min.

Restriction fragments were compared by designating and recording 50 fragment positions using an equidistant marker. Each gel included two control lanes for the reference isolate *A. flavus* genotype GT 36, as represented by K. E. Papa strain NRRL 19997 (17,30) and a Lambda standard. Fragments within and between gels could be distinguished using the reference isolate. Strains producing similar banding patterns in different gels were reprobated on the same gel to verify fragment positions. Some thicker and darker bands were difficult to decipher, and these were reprobated, in addition to using different film exposures to identify bands of varying intensity. Fragments of more than 20 kb and less than 1.5 kb were not included in the analyses. *A. parasiticus* genotypes (pAF28 fingerprints) were classified on the basis of the presence or absence of fragments, each of which is presumed to represent a single genetic locus. Isolates with identical fingerprints are recognized as belonging to the same genotype and may represent the same clonal population. Isolates sharing more than 80% of the fragments in common were recognized as belonging to the same fingerprint group, while strains sharing 80% or fewer fragments were clas-

sified as separate fingerprint groups after Xia et al. (32).

Analysis of genotype relatedness. DNA banding patterns generated by restriction fragment length polymorphisms (RFLP) of total genomic DNA were evaluated; each hybridizing DNA band was considered a discrete character, and each character has two possible states, scored 0 and 1 for absence and presence of that character, respectively. Included in the phenogram are three *A. flavus* reference strains (GT 36 = NRRL 19997, GT 09 = NRRL 27673, GT 10 = NRRL 27672) representing the most frequently recorded RFLP fingerprint groups isolated from the IRVSF (30). Dendrograms were constructed based on RFLP using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc Version 2.01, Exeter Software, Setauket, NY) (25). With NTSYS-pc analysis, matrices based on Dice similarity coefficients (C) were used to transform the character matrices into simple pair-wise matching similarity values and were calculated for each pair of isolates, where $C = 2N_{xy}/(N_x + N_y)$; N_{xy} is the number of hybridizing DNA bands shared by the isolates x and y, and N_x and N_y refer to the numbers of DNA bands in isolates x and y, respectively. The similarity coefficients were used to generate the cluster analysis and were computed in the SIMQUAL program. The phenogram was

Fig. 1. DNA fingerprints of selected *Aspergillus parasiticus* isolates from soil samples collected from corn plantings in a field near Kilbourne, Illinois. The figure includes one reference strain (GT 36 = *Aspergillus flavus* NRRL 19997) and size markers.

Fig. 2. Phenogram based on cluster analysis of 60 isolates of *Aspergillus parasiticus* and 3 *Aspergillus flavus* reference strains (NRRL 19997, NRRL 27672, NRRL 27673) using the Dice similarity coefficient of individual DNA bands produced by hybridization with pAF28 repetitive sequence (17). All isolates produced sclerotia except those marked with a minus (-) symbol. The phenogram was generated using the NTSYS-pc Version 1.8 (25).

generated by the unweighted pair-group arithmetic average method (UPGMA) in the SAHN clustering program. The SAHN clustering program enables us to efficiently compare the DNA banding patterns of *A. flavus* and *A. parasiticus* strains to determine which strains share identical fingerprints or can be classified in the same fingerprint group. This clustering program is not a method for establishing phylogenetic trees (25).

RESULTS AND DISCUSSION

Populations of *A. flavus* and/or *A. parasiticus* for soil samples collected from five different corn plantings within the IRVSF ranged from 0.2 to 4.0 CFU/g of dry weight soil (maximum of total filamentous fungi reached < 50,000 CFU/g). These values for a sandy soil type are considerably lower than 396 to 1,200 CFU of *A. flavus* and *A. parasiticus* per g for corn fields in eastern Iowa (26) and 100 to 270 CFU/g for corn cropping systems at Columbia, Missouri (1), where the total filamentous fungi may exceed 1 million CFU/g of dry weight soil. Although *A. flavus* and *A. parasiticus* were reported in near equal numbers from corn field soils, corn ears are infected primarily by *A. flavus* (1,13,15,23,33).

Sixty isolates of *A. parasiticus* were obtained from platings of IRVSF soil samples. Pair-wise combinations of isolates revealed DNA banding patterns with similarity values of 11 to 100%. In the present paper, isolates with 80% similarity were clustered together and considered a distinct fingerprint group, while isolates showing 100% band similarity are recognized as belonging to the same genotype and possibly the same clonal population. In an earlier paper, we referred to DNA fingerprint groups as genotypes (30). Thirty-three distinct DNA fingerprint groups (33 fingerprint groups/60 isolates = 55%) were identified, with each group sharing less than 80% pAF28 band similarity, and including 50 (83%) distinct genotypes sharing less than 100% pAF28 band similarity (Figs. 1 and 2). Furthermore, 42 (84%) of the 50 *A. parasiticus* genotypes were from single isolates. These estimates of DNA fingerprint diversity and genotype diversity for 60 *A. parasiticus* isolates were equivalent to estimates of 81% genotype diversity for a population of 31 *A. flavus* isolates from the same soil samples, but lower than estimates of 77% DNA fingerprint group diversity (30). While no dominant clone was detected, one fingerprint group including NRRL 26723 represented 13% of the population.

Horn and Greene (12) reported only 22% VCG diversity for the *A. parasiticus* population (76 isolates) from a peanut field, whereas the diversity of *A. parasiticus* fingerprint groups (RFLP) at the IRVSF was 55%. The greater genetic homogeneity of the Georgia population in-

vides further interpretation: (i) The individual VCG groups could include isolates that would produce unique DNA fingerprints using the pAF28 repetitive sequence. McAlpin and Mannarelli (17) showed that the pAF28 repetitive sequence could distinguish among tester strains of *A. flavus* belonging to the 22 VCGs determined by Papa (20) and also produced hybridization patterns that could distinguish among isolates of *A. parasiticus*. (ii) There were numerous "repeat" isolates of the same VCG because three *A. parasiticus* isolates were selected from each of 18 soil samples (14). (iii) Because *A. parasiticus* infects peanuts, the peanut crop could selectively impact VCG clonal densities in field soil.

RFLP analysis of nuclear DNA was shown to distinguish *A. parasiticus* from *A. flavus* and identify "subgroups" among the isolates representing these species, although a number of isolates could not be independently differentiated (18). A UPGMA cluster dendrogram constructed from nucleotide sequence divergence values (*p* values) produced subgroupings which, apart from a population of *A. flavus* isolates from southern India, did not consistently correlate with geographical origin or aflatoxin-producing ability. Bayman and Cotty (5) reported that both cladistic and phenetic analysis distinguished two *A. parasiticus* isolates from 26 isolates of *A. flavus* when random amplified polymorphic DNAs (RAPDs) were used to estimate genetic distance between isolates. In the present study, the phenetic analysis and phenogram (Fig. 2) separates 60 *A. parasiticus* isolates from three *A. flavus* reference strains. However, when fingerprints from these same 60 *A. parasiticus* strains were evaluated along with fingerprints from 269 *A. flavus* strains isolated from IRVSF (30), the phenetic analysis did not consistently separate strains according to species (C. E. McAlpin and D. T. Wicklow, unpublished data). Although the DNA probe pAF28 has proven useful in differentiating among closely related strains of *A. flavus* group species, including strains belonging to genetically isolated VCGs, the exact nature, extent, and source of the variability detected is not known (17).

Seventy-eight percent of *A. parasiticus* isolates from this study produced sclerotia during dark incubation at 25°C. Among 13 isolates that failed to produce sclerotia, five belonged to the same fingerprint group, and two of the genotypes (100% band similarity) included both sclerotium producers and nonproducers (Fig. 2), as earlier reported for *A. parasiticus* VCGs (14). Bayman and Cotty (5) noted that complementation tests for vegetative compatibility were not always a good indicator of relatedness, identifying one example where different sclerotial morphs, distinguished by sclerotium diameter and numbers of sclerotia per area of colony surface, were found in *A. flavus* isolates belonging

to the same VCG. In the present study, each of the 60 *A. parasiticus* isolates from IRVSF soils produced aflatoxins B₁ and B₂, and G₁ and G₂, while 36% of the 31 *A. flavus* isolates from the same soil samples produced aflatoxins B₁ and B₂ (30). No population of *A. parasiticus* has been shown to include significant numbers of non-aflatoxin-producing strains (1,14,16).

First described as a pathogen of mealy bugs of sugarcane (*Saccharum officinarum* L.) in Hawaii (28), *A. parasiticus* attained notoriety when it was shown to be responsible, in part, for aflatoxin contamination in peanuts (*Arachis hypogaea* L.) (3,9). Reports of *A. flavus* and *A. parasiticus* in northern latitudes are primarily associated with stored grain (7), forage (27), or diseased insects (6,24). Isolates of *A. parasiticus* from nonagricultural habitats in northern latitudes are few (e.g., CMI 297541, loess near Loveland, Iowa; NRRL A-24120, seed of yellow coneflower, Peoria, Illinois; NRRL 13005, microarthropod in Beech forest litter, Berrien County, Michigan) and include reported isolations from eastern Europe (e.g., steppe soil, uranium mine, and feathers of free-living birds) (10). There are no records of *A. flavus* or *A. parasiticus* from prairie soils in Missouri, Iowa, or Wisconsin (1,19,26). The regular occurrence of *A. flavus* and *A. parasiticus* in agricultural soils from the midwestern corn belt can be attributed to soil disturbance through cultivation and soil incorporation of nutrient-rich crop residues that support fungal saprotrophic growth (4,11). The genotypic diversity of the *A. flavus* and *A. parasiticus* population at IRVSF could be enriched by strains entering the field from surrounding forest and prairie habitats. It was not our objective to contrast the genetic relatedness of geographically separated populations of *A. parasiticus*.

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