Aspergillus parasiticus Communities Associated with Sugarcane in the Rio Grande Valley of Texas: Implications of Global Transport and Host Association Within *Aspergillus* Section *Flavi*

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ABSTRACT

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In the Rio Grande Valley of Texas (RGV), values of maize and cottonseed crops are significantly reduced by aflatoxin contamination. Aflatoxin contamination of susceptible crops is the product of communities of aflatoxin producers and the average aflatoxin-producing potentials of these communities influence aflatoxin contamination risk. Cropping pattern influences community composition and, thereby, the epidemiology of aflatoxin contamination. In 2004, *Aspergillus parasiticus* was isolated from two fields previously cropped to sugarcane but not from 23 fields without recent history of sugarcane cultivation. In 2004 and 2005, *A. parasiticus* composed 18 to 36% of *Aspergillus* section *Flavi* resident in agricultural soils within sugarcane-producing counties. *A. parasiticus* was not detected in counties that do not produce sugarcane. *Aspergillus* section *Flavi* soil communities within sugarcane-producing counties

differed significantly dependent on sugarcane cropping history. Fields cropped to sugarcane within the previous 5 years had greater quantities of *A. parasiticus* (mean = 16 CFU/g) than fields not cropped to sugarcane (mean = 0.1 CFU/g). The percentage of *Aspergillus* section *Flavi* composed of *A. parasiticus* increased to 65% under continuous sugarcane cultivation and remained high the first season of rotation out of sugarcane. Section *Flavi* communities in fields rotated to non-sugarcane crops for 3 to 5 years were composed of <5% *A. parasiticus*, and fields with no sugarcane history averaged only 0.2% *A. parasiticus*. The section *Flavi* community infecting RGV sugarcane stems ranged from 95% *A. parasiticus* in billets prepared for commercial planting to 52% *A. parasiticus* in hand-collected sugarcane stems. Vegetative compatibility assays and multilocus phylogenies verified that aflatoxin contamination of raw sugar was previously attributed to similar *A. parasiticus* in Japan. Association of closely related *A. parasiticus* genotypes with sugarcane produced in Japan and RGV, frequent infection of billets by these genotypes, and the ephemeral nature of *A. parasiticus* in RGV soils suggests global transport with sugarcane planting material.

In the Rio Grande Valley in South Texas (RGV), aflatoxin concentrations are the most important criterion dictating the marketability and value of locally produced cottonseed and maize (66,89). Aflatoxin contamination is caused by several species in *Aspergillus* section *Flavi* (13) which vary widely both within and among species in capacity to synthesize these highly carcinogenic toxins (5,24,27). Some species produce both B and G aflatoxins while others produce only B aflatoxins due to a 0.9- to 2-kb deletion in the aflatoxin biosynthesis gene cluster (25,72). Based on genetic, physiological, and cultural characteristics, these species can be subdivided into distinct morphotypes (17) and vegetative compatibility groups (VCGs) (4). Diverse species, strains, morphotypes, and VCGs exist in complex communities that vary widely in average aflatoxin-producing ability among regions and fields (2,5,69,90). The average aflatoxin-producing potential of *Aspergillus* section *Flavi* communities is a central criterion dictating the potential for crop aflatoxin contamination (47). *Aspergillus flavus* and *A. parasiticus* are the fungi most associated with aflatoxin contamination (51). However, *A. flavus* isolates vary widely in aflatoxin production, with some isolates producing little or no aflatoxins. *A. parasiticus* typically produces more consistent and higher concentrations of aflatoxins (22,88).

Aflatoxin contamination may result from infection by aflatoxin-producing fungi both during crop development and after crop maturation (17,76). Although it is clear that the aflatoxin-producing potential of fungal communities associated with crops influences the likelihood of aflatoxin contamination, little is known about interactions between community composition of aflatoxin-producing fungi and crop plant cultivation (16). A few studies suggest that compositions of *Aspergillus* communities in field soils are influenced by both current and preceding crops (43,48,65).

A. parasiticus is an antagonist of sugarcane mealybugs in Hawaii, the Philippines, Australia, and Colombia (23,31,87,93) and, in this context, this species has been repeatedly associated with sugarcane. In Japan, fungi morphologically intermediate but closely related to both *A. parasiticus* and *A. flavus* were isolated from sugarcane in connection with aflatoxin contamination of raw sugar (54,80,82,83). Although the pink sugarcane mealybug is rarely observed in Texas (61,62), the role of sugarcane production in shaping *Aspergillus* section *Flavi* communities has not been explored. In RGV, sugarcane fields neighbor those of cotton and maize, with typical agronomic practice cropping sugarcane for 3 to 5 years with a single yearly harvest. Cotton typically follows sugarcane cropping and alternates with maize or sorghum in the following seasons.

The current study evaluated influences of sugarcane cultivation in Texas on compositions of *Aspergillus* section *Flavi* communities. In addition, *A. parasiticus* isolates from Texas sugarcane fields were subjected to phylogenetic and vegetative compatibility

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analyses along with isolates from Asia and elsewhere in order to investigate sugarcane-associated *A. parasiticus* lineages. The results suggest association and distribution of a distinct lineage of *A. parasiticus* with sugarcane cultivation in both Asia and North America.

MATERIALS AND METHODS

Analysis of fungal communities in agricultural fields of the RGV. In 2004, communities of *Aspergillus* section *Flavi* resident in soils of 14 agricultural fields planted to either maize or cotton were analyzed to determine the relative prevalence of various aflatoxin-producing species. Fields were distributed across 25 km² and contained within SRS Farms (Mercedes, TX) that provided cropping histories for each field. One to two 150- to 200-g soil samples were taken from each field. Soil samples consisted of 30 to 40 subsamples taken from the top 2 cm of soil along a 40- to 50-m transect (45,48). In 2005, 64 fields with varying histories of sugarcane cropping were sampled throughout the RGV in order to test specific association of *A. parasiticus* with sugarcane cultivation. Fields were chosen that had never cropped sugarcane, had cropped sugarcane for <1 year, had cropped sugarcane for >1 year, and those rotated to crops other than sugarcane for 1 year or 3 to 5 years after sugarcane cropping. Continuous sugarcane cropping ($n = 21$) and recent rotation out of sugarcane ($n = 6$) were considered the same treatment because the association between sugarcane and *A. parasiticus* was first observed in 2004 in a cotton field cropped to sugarcane in 2003. In April, the northern portion (21 fields) of the sugarcane production area was sampled. In June, eight additional fields in the northern portion of the sugarcane-growing region were sampled as well as fields in the eastern ($n = 5$), western ($n = 8$), and southern ($n = 17$) portions of the RGV sugarcane region. In both April and June, fields were selected based on the recorded history of sugarcane cultivation over the previous 10 years. Cropping information was provided by Rio Grande Valley Sugar Growers, Inc. (RGVSG, Santa Rosa, TX).

Soil samples were dried in a forced-air oven (Sheldon Manufacturing, Cornelius, OR) at 45°C for 48 h, pulverized by hammering, homogenized by repeated mixing, and transferred to zippered plastic bags. Samples were stored at room temperature until use. Sample suspensions were made by stirring (300 rpm) 10 to 20 g of homogenized soil in 50 ml of sterilized distilled, deionized water for 10 to 15 min with a magnetic stir bar. Soil suspension (100 to 500 μ l/dish) was spread evenly across the surface of rose Bengal agar modified to favor identification of *Aspergillus* section *Flavi* (11) and incubated (darkness, 31°C, 3 days). After incubation, *Aspergillus* section *Flavi* colonies were counted to calculate CFU. Discrete colonies from plates with ≤ 10 colonies were transferred to 5% V8 juice medium (13) with sterile wooden sticks. After incubation (darkness, 31°C, 5 to 7 days), agar plugs of sporulating colonies were transferred to sterile water (2 ml in 5-ml vial) and stored at 4°C (10). All colonies on a plate were counted and at least 15 *Aspergillus* section *Flavi* colonies were obtained from each (13).

Plant samples. Sugarcane billets were provided by RGVSG. As components of normal sugar production in RGV, billets (stem pieces) were harvested from 15 RGV locations, prepared for use in commercial plantings, and shipped directly from RGVSG to the School of Plant Sciences, University of Arizona, Tucson. Each billet sample consisted of 10 billets, 40 to 60 cm in length. Billet samples were surface sterilized by spraying with 80% ethanol solution and aseptically air dried in a laminar flow hood (NuAire, Plymouth, MN). Additional sugarcane stem samples were collected by hand in Hidalgo (21 fields) and Cameron (6 fields) Counties just prior to harvest in 2005. Mature stalks at least 3 cm in diameter were selected for sampling; a single piece (30 to 45 cm long) from each stalk was cut to include the crown at the

soil surface. Samples were selected close to the ground because *A. parasiticus* had been frequently isolated from soils. Billet and hand-collected stem pieces were placed in a forced-air drying oven 45°C for 1 to 2 days, minced with a cutting mill (IKA Labortechnik; IKA Werke GmbH & Co. KG, Staufen, Germany), and stored at room temperature in plastic bags until subjected to dilution plating technique. Homogenized sample (≈ 5 g) was added to a 7-ml sterile polystyrene tube containing 5 ml of 0.01% Tween 80 in distilled water. The resulting suspension was mixed for 20 min by rotation (150 rpm, Roto Shake Genie; Scientific Industries, Bohemia, NY). Aliquots (100 μ l) of the suspension and a 1:10 dilution in 9 ml of Butterfield's buffer (Hardy Diagnostics, Santa Maria, CA 93455) were subsequently spread aseptically onto three modified rose Bengal agar plates (13). If >10 *Aspergillus* section *Flavi* cultures were detected on the isolation plates, the quantity of ground sample was decreased to 1 g and further dilutions were performed, including 1:100 or 1:1,000 serial dilutions of 1-ml sample suspensions.

Data analysis. The study area was ≈ 100 km wide by 400 km long, extending from the eastern Texas–Tamaulipas international border in the south to Jackson County in the north. Data were analyzed separately for each county. Quantities of *A. parasiticus*, *A. flavus*, and *A. tamarii* in soil were calculated as the number of CFU g⁻¹. The *Aspergillus* section *Flavi* community was characterized for each plant sample as the proportion of the *Aspergillus* section *Flavi* community occupied by each species. Community composition was calculated by dividing the number of isolates of each species by the total number of *Aspergillus* section *Flavi* isolates and multiplying by 100. Analysis of variance using general linear models was used to assess effects of county and cropping history on *Aspergillus* section *Flavi* community composition and CFU g⁻¹.

Fungal isolates. Thirty-two fungal isolates belonging to *Aspergillus* section *Flavi* were used for phenotypic and phylogenetic comparison (Table 1). Isolates were chosen from laboratory and public culture collections to show maximum genetic diversity within the section, and include reference strains and type isolates from *A. nomius*, *A. flavus*, and *A. parasiticus* species (30). In all, 10 putative *A. parasiticus* isolated in association with sugarcane in RGV and 3 from Japan were also included. Cultures were grown in the dark at 31°C on 5-2 medium (10). Isolates were stored on 5-2 (10) 3-mm modified V8 agar plugs in sterile distilled water at 4°C.

Morphology. After incubation (5-2 agar, 31°C, 7 days, darkness) (10), colonies were identified by macroscopic colony and microscopic ($\times 400$) conidia characteristics (52,55). Isolates with olive-brown to brown near-coffee colonies with thick-walled, distinctly roughened conidia were identified as *A. tamarii*. *A. flavus* L strains were most frequently grayish-green to olive with some colonies moss green in color (52,53). *A. parasiticus* obtained from RGV were colored deep green, olive green, or olive (52,53). *A. flavus* and RGV *A. parasiticus* with similar colony coloring were distinguished by conidial wall ornamentation. Fungi with smooth-surfaced conidia and no ornamentation at $\times 400$ were identified as *A. flavus*. Fungi with conidial ornamentation ($\times 400$ magnification) within the range reported for the species (52,54) were identified as *A. parasiticus*. Isolates were also cultured on *Aspergillus flavus* and *parasiticus* agar (AFPA) medium (31°C, 5 days) and development of a bright orange reverse was confirmatory for *A. parasiticus* or *A. flavus* (11,70).

Aflatoxin production assay. Aflatoxin production was assayed in a modified Adye and Mateles medium made in 50 mM MES buffer (Research Organics, Cleveland), pH 6.15, containing sucrose and ammonium sulfate as the sole carbon and nitrogen sources, respectively (13,60). Spore suspension was seeded into 250-ml Erlenmeyer flasks containing 70 ml of medium and incubated on an orbital shaker (31°C, 5 days, darkness, 150 rpm). After incubation, the pH of each culture was measured, and 50 ml

of acetone was added to each flask to lyse the cells and solubilize the aflatoxin (13). Two 4- μ l subsamples of acetone/culture suspension were spotted directly onto thin-layer chromatography (TLC) plates (TLC silica gel 60, 20-by-20-cm glass plates; EMD Chemicals, Inc., Gibbstown, NJ) beside aflatoxin standards (Sigma-Aldrich, St. Louis). Plates were developed in 96% ethyl ether, 3% methanol, and 1% distilled deionized water (9); dried, examined under an UV light (365 nm); and scored for presence of B aflatoxins or B and G aflatoxins or aflatoxins below the limit of detection (1 mg/kg).

Vegetative compatibility analyses. Vegetative compatibility analyses were performed to determine the intimacy of relatedness of *A. parasiticus* isolated from sugarcane and field soils in RGV and Japan. Nitrate-nonutilizing auxotrophs were generated for three *A. parasiticus* isolates (OPS485, OPS500, and OPS515) (54) from Japanese sugarcane on chlorate medium, as described previously (12,54). Briefly, spore suspensions were seeded into a 3-mm-diameter well in the center of a petri dish containing SEL agar (Czapek-Dox broth with 25 g of KClO₃, 50 mg of Rose Bengal, and 20 g of Bacto agar [Difco Laboratories, Detroit] per liter, pH 7.0) (12). Auxotrophic sectors formed spontaneously during incubation (31°C, 7 to 45 days, darkness) were transferred to MIT medium (Czapek-Dox broth with 15 g of KClO₃ and 20 g Bacto agar [Difco Laboratories] per liter) to stabilize the mutants and eliminate wild-type mycelia (12). Nitrate auxotrophs (*nit*⁻ mutants) were then phenotyped on media containing NO₃, NO₂, or hypoxanthine as sole nitrogen sources, and mutant tester pairs consisting of complimentary *cnx*⁻ and *niaD*⁻ mutants were developed for each isolate (3). The *nit*⁻ mutants from sugarcane-associated *A. parasiticus* isolates from RGV were paired on starch medium (3.0 g of NaNO₃, 1.0 g of K₂HPO₄, 0.5 g of MgSO₄, 0.5 g of KCl, 36 g of dextrose, and 1.0 ml of A&M micronutrients per liter) (18) with the tester pairs for each Japanese isolate to assess potential membership (4). Plates were incubated (31°C, 10 days, darkness) and VCG membership was

indicated by auxotroph complementation resulting in wild-type growth in the zone of mycelial interaction between at least one tester and the isolate *nit*⁻ mutant. The tester pair for the most common VCG, OPS500, was deposited at the Fungal Genetics Stock Center (accession numbers FGSC A1915 and A1916).

DNA isolation and polymerase chain reaction amplification.

For the following procedures, isolates previously transferred by single spore were cultured on 5-2 agar (31°C, 5 days, darkness) (10). After incubation, spores were collected with a sterile cotton swab and suspended in sterile distilled water. The resultant spore suspension (100 μ l) was added to 70 ml of sterile potato dextrose broth (Difco Laboratories) in a 250-ml Erlenmeyer flask and incubated on a rotary shaker (5 days, 31°C, darkness, 150 rpm). Mycelia were captured on Miracloth (EMD Millipore, Billerica, MA) and DNA was isolated using the FastDNA SPIN Kit and the Fast Prep Instrument (Qbiogene, Inc. Carlsbad, CA). DNA was stored in buffer at either -20 or -80°C (long-term). Three genes (*ITS1*, *aflR*, and *niaD*) were directly sequenced. Polymerase chain reaction (PCR) of internal transcribed spacer (*ITS1*) used the primers ITS4 and ITS5 (435 bp), as previously described (92). A portion of the gene encoding the aflatoxin transcription factor *aflR* (1.6 kb) was amplified in three pieces using primers AFLR-F (5'-GGAAACAAGTCTTTCTGG-3') and AFLR-R (5'-CAGA GCGTGTGGTGGTTGAT-3'), AFLR1F (5'-AGAGAGCCAACT GTCGGACCAA-3') and AFLR1R (5'-GGGTGACCAGAGAAC TGCGTGAT-3'), and AFLR2F (5'-GACCTCCGGCGCATAACA CGTA-3') and AFLR2R (5'-ACGGTGGCGGGACTGTTGCTA CA-3') (27). *aflR* primers did not work well for three Asian *A. parasiticus* isolates. For these isolates, the entire *aflR* region was amplified using primers AFLJR1F (5'-CAT GGC TGA GGA TAG CTC GTG-3') and AFLJR20R (5'-GTG TGT TGA TCG ATC GGC CAG-3') (27). The resulting amplicons were then successfully used as the template for the three initial *aflR* primer pairs. A portion of the nitrate reductase gene (2.2 kb), *niaD*, was amplified in three pieces using the primers NIADF (5'-CGGACGATAA

TABLE 1. *Aspergillus* section *Flavi* isolates used in this study

Isolate	Other names	Species	Location ^z	Substrate	Citation	Aflatoxins
NRRL 13137	M93, ATCC 15546	<i>nomius</i>	Illinois	Wheat	55	BG
AF36	YV36	<i>flavus</i>	Arizona	Cottonseed	10	None
V	MR17	<i>flavus</i>	Arizona	Cottonseed	10	B
AF13	ATCC 96044	<i>flavus</i>	Arizona	Citrus soil	10	B
P19	YV19	<i>flavus</i>	Arizona	Bermuda grass soil	10	B
AF12	ATCC MYA-382	<i>flavus</i>	Arizona	Cotton field soil	10	B
AF42	ATCC MYA-383	<i>flavus</i>	Arizona	Cottonseed	10	B
AF70	ATCC MYA-384	<i>flavus</i>	Arizona	Cotton field soil	10	B
OPS417	...	<i>parasiticus</i>	Vietnam	Peanut	54	BG
NRRL 2999	ATCC 26691	<i>parasiticus</i>	Uganda	Peanut	75	BG
OPS393	...	<i>parasiticus</i>	Vietnam	Peanut	54	BG
BN009E	...	<i>parasiticus</i>	Benin	Mixed crop soil	70	BG
CP461	ATCC-62882	<i>parasiticus</i>	Georgia	Peanut	22	None
NRRL 424	...	<i>parasiticus</i>	Georgia	Peanut field soil	68	BG
OPS651	...	<i>parasiticus</i>	China	Peanut	54	BG
NRRL 4123	...	<i>parasiticus</i>	Georgia	Maize	68	BG
SU1	ATCC 56775	<i>parasiticus</i>	Uganda	Peanut	56	BG
NRRL 465	CBS 571.65	<i>parasiticus</i>	Unknown	Unknown	91	BG
NRRL 502	...	<i>parasiticus</i>	Hawaii	Mealybug	68	BG
A17-H	...	<i>parasiticus</i>	RGV	Sugarcane field soil	Current study	BG
A17-N	FGSC A1921	<i>parasiticus</i>	RGV	Sugarcane field soil	Current study	BG
Gal5-N	...	<i>parasiticus</i>	RGV	Sugarcane field soil	Current study	BG
Gal22-J	FGSC A1917	<i>parasiticus</i>	RGV	Sugarcane field soil	Current study	BG
Har1-AC	...	<i>parasiticus</i>	RGV	Cotton lint	Current study	BG
Har7-O	FGSC A1918	<i>parasiticus</i>	RGV	Cotton lint	Current study	BG
Mad1-B	...	<i>parasiticus</i>	RGV	Sugarcane field soil	Current study	BG
Mad2-H	FGSC A1919	<i>parasiticus</i>	RGV	Sugarcane field soil	Current study	BG
OPS485	...	<i>parasiticus</i>	Japan	Sugarcane field soil	54	BG
OPS500	...	<i>parasiticus</i>	Japan	Sugarcane field soil	54	BG
OPS515	...	<i>parasiticus</i>	Japan	Sugarcane field soil	54	BG
Ray2-F	...	<i>parasiticus</i>	RGV	Sugarcane field soil	Current study	BG
Ray4-C	...	<i>parasiticus</i>	RGV	Sugarcane field soil	Current study	BG

^z RGV = Rio Grande Valley of Texas.

GCAACAACAC-3') and NIADR (5'-GAGCCGTTACATTCTCACAC-3'), NIADBF (5'-ACGGCCGACAGAAGTGCTGA-3') and NIADBR (5'-ACGGGGAGTCTCTTCGCCCA-3'), and NIAD3F (5'-GTCACACGGGCACATCTA-3') and NIAD3R (5'-ATGCCTACAGGATGGATG-3'). PCR reactions were carried out in 20 µl of AccuPower HotStart PCR PreMix polypropylene tube strips (Bioneer Corporation, Daejeon, Korea) using 18 µl of autoclaved, distilled, deionized water and 1 µl of genomic DNA (15 ng/µl final DNA concentration). PCR conditions for *ITS* primers included a denaturation and hot start step of 5 min at 94°C; followed by 35 cycles of 94°C for 20 s, 54.3°C for 20 s, and 72°C for 30 s; and final extension at 72°C for 10 min. PCR conditions for *afIR* primers were similar, except the extension was 90 s and annealing was at 47°C for AFLR F-R, 59°C for AFLR 1F-1R, 57°C for AFLR 2F-2R, and 55°C annealing and 2.5-min extension for AFLJR 1F-20R. PCR conditions for the *NiaD* region were similar, except annealing temperatures were 52°C for primers NIAD F-R and NIAD 3F-3R and 57°C for primers NIAD BF-BR. Amplicons were separated by electrophoresis on 1% agarose gels and evaluated for size and singularity. Excess primers and unincorporated nucleotides were degraded with ExoSAP-IT (USB Corporation, Cleveland) (1 µl of ExoSAP-IT in 16 µl of PCR product at 37°C for 1 h, followed by 85°C for 15 min). Purified PCR products were sequenced twice (once in each direction) by the University of Arizona sequencing facility, UAGC, with a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Novel *A. parasiticus* sequences were submitted to the National Center for Biotechnology Information GenBank with accession numbers KC769488–KC769508 for *afIR* and KC782772–KC782791 for *niaD*.

Phylogenetic analyses. Sequences were edited and aligned using Geneious Pro (version 4.8.2; Biomatters Ltd., Auckland, New Zealand). The three sugarcane-associated *A. parasiticus* isolates from Japan as well as two isolates from each of six RGV sampling sites were included in the phylogenetic analyses. Although, *A. parasiticus* isolates directly isolated from sugarcane plant samples were not included in the phylogenies, several Texas isolates were included that were known to belong to VCGs isolated repeatedly from sugarcane tissues. Two *A. parasiticus* isolates from cotton lint collected from RGV fields previously cropped to sugarcane were also included. JModelTest 0.1 was used to identify the model of nucleotide substitution that best fit the data from each locus (71). Phylogenetic trees were constructed with both maximum-likelihood (ML) (Phylip version 3.68) (29), and maximum-parsimony (MP) (Paup 4.0b10) (81) methods. MP analyses utilized heuristic searches with random stepwise addition, tree bisection-reconnection branch swapping, and 100 replicates. Gaps were treated as missing data. Branch

supports for all analyses were estimated using 1,000 bootstrap replicates, with a heuristic search consisting of 100 random-addition replicates for each bootstrap replicate. All characteristics were unordered and equally weighted.

RESULTS

Association of *A. parasiticus* with sugarcane. Among 158 agricultural fields sampled in an area extending from the Upper Coast through RGV in 2004 and 2005, *A. parasiticus* was only recovered from soils in sugarcane-producing RGV counties and was never isolated outside sugarcane-producing counties (limit of detection = 7% within fields and 0.1% across non-sugarcane fields) (Table 2). Within three sugarcane-producing counties, proportions of *Aspergillus* section *Flavi* communities comprising *A. parasiticus* in fields were 0 to 100%. Abundance of *Aspergillus* section *Flavi* fungi in soil (CFU g⁻¹) differed with sugarcane cropping history ($t_{1,56} = 5.50$, $P < 0.0001$) and varied among counties regardless of sugarcane cropping ($F_{5,152} = 26.70$, $P < 0.0001$). Sugarcane-cropping counties yielded the fewest section *Flavi* fungi, with fungi in fields at 2.66 to 323.8 CFU g⁻¹ (Table 2). Counties that did not crop sugarcane yielded *Aspergillus* section *Flavi* fungi at 10.7 to 4,400.7 CFU g⁻¹.

The association of *A. parasiticus* with sugarcane cultivation was consistent over the 2 years studied in RGV (Table 3). In Hidalgo County in 2004 and on two dates in 2005, *A. parasiticus* was found only in the 21 fields cropped to sugarcane in the previous 5 years (Table 3). No *A. parasiticus* was detected in the 42 fields not cropped to sugarcane in the previous 5 years. *A. parasiticus* was detected only in one field in Cameron County 2005 sampling without a history of sugarcane (4.8% *A. parasiticus*) (Table 3). Differences in frequencies of *A. parasiticus* between sugarcane and non-sugarcane fields were consistent in both April and June within sugarcane-producing counties (Table 3). *A. flavus* CFU/g of soil was similar in both counties and both sugarcane and non-sugarcane fields (Table 3).

The proportion of the *Aspergillus* section *Flavi* community comprising *A. parasiticus* was dependent upon cropping history, with both the duration of sugarcane cropping and the number of years out of sugarcane significantly influencing the percent *A. parasiticus* ($F_{3,74} = 51.23$, $P < 0.0001$) (Fig. 1). Percent *A. parasiticus* was greatest in the 27 fields either <2 years out of sugarcane or with one or more sugarcane harvests. Analysis of similarity testing revealed that the *Aspergillus* section *Flavi* communities of fields in continuous sugarcane cropping and those rotated to other crops for <2 years were not statistically different ($R = -0.1822$, $P = 0.97$); therefore, these two potential treatments were pooled in the analyses. Ten fields planted to sugarcane but

TABLE 2. *Aspergillus* section *Flavi* communities in Texas counties that do or do not crop sugarcane

Region, Texas county	Sugarcane cropping ^v	Fields ^w	Isolates ^x	CFU ^y	<i>Aspergillus parasiticus</i> (%) ^z
Rio Grande Valley					
Cameron	Yes	17	416	69 c,d	30 a
Hidalgo	Yes	62	1,421	49 d	18 a
Willacy	Yes	5	75	26 b,c,d	36 a
Total	Yes	84	1,912	48 B	21 A
Coastal Bend					
Calhoun	No	10	563	1,289 a	ND b
Jackson	No	27	418	289 b	ND b
San Patricio	No	37	2,224	202 b,c	ND b
Total	No	74	3,205	593 A	ND B

^v Yes indicates sugarcane was commercially cropped in the county within the past 25 years.

^w Composite soil samples (≈200 g) composed of 30 to 40 subsamples along an 80- to 100-m diagonal were taken from each field (1 to 6 samples/field). In counties with sugarcane cropping, both fields that had been recently cropped to sugarcane and fields without sugarcane for the past 5 years.

^x Only *Aspergillus* section *Flavi* fungi were enumerated and isolated (at least 12 isolates from each soil sample).

^y CFU were calculated per gram of soil. CFU with a common letter (lowercase) are not significantly different ($P < 0.05$) by Fisher's protected least significant difference (LSD) test; totals not followed by the same letter (A or B) are significantly different by Student's *t* test (two sided; $P < 0.0001$).

^z ND = *A. parasiticus* not detected (limit of detection = 7%). Percent *A. parasiticus* was significantly different among treatments ($F_{5,152} = 6.08$, $P < 0.0001$). Percentages not followed by the same letter (a, b, A, or B) are significantly different using Fisher's protected LSD test.

sampled prior to the first harvest had a similar percent *A. parasiticus* as six fields rotated to non-sugarcane crops for 3 to 5 years. *A. parasiticus* communities in fields 3 to 5 years out of sugarcane also were not statistically different from fields never cropped to sugarcane. Cropping history also influenced the proportion of the *Aspergillus* section *Flavi* community comprising *A. flavus* ($F_{3,74} = 54.78$, $P < 0.0001$), with *A. flavus* dominating fields without recent sugarcane cropping (Fig. 1).

Overall, *A. parasiticus* was the member of *Aspergillus* section *Flavi* most frequently associated with both sugarcane billets obtained from the RGVSG sugar mill and plant stems harvested by hand (Table 3). *A. parasiticus* was present in every plant sample taken but *A. flavus* was not detected in 13 of 77 plant samples. One *A. tamarii* isolate (<0.01% of the total *Aspergillus* section *Flavi* community) was recovered from a field-collected sugarcane sample. Billet samples from three counties had similar *Aspergillus* section *Flavi* communities, with *A. parasiticus* percentages higher than *A. flavus* percentages (Table 4). Hand-harvested stems had a greater percentage of *A. flavus* than commercial billet samples (Table 4). The community in hand-collected stems differed by county, with *A. flavus* dominating in Cameron County and *A. parasiticus* dominating samples from Hidalgo (Table 4). Hand-collected plant samples and billets were not symptomatic of any disease and were not infested by pink sugarcane mealybugs.

Sugarcane-associated *A. parasiticus* isolates from Japan and the United States are distinct from other *A. parasiticus* isolates. Based both on morphology and DNA sequence data, *A. parasiticus* from Texas sugarcane fields was more similar to *A. parasiticus* from Japanese sugarcane fields than to either *A. flavus* or *A. parasiticus* not associated with sugarcane. The three *A. parasiticus* isolates from Japanese sugarcane were morphologically indistinguishable from RGV *A. parasiticus*. Colony color and spore ornamentation were similar to that for non-sugarcane-associated *A. parasiticus* but non-sugarcane-associated *A. parasiticus* produced sclerotia on Czapek's medium whereas sugarcane-associated *A. parasiticus* did not, although sclerotia production is an unreliable taxonomic characteristic (30). All RGV *A. parasiticus* isolates assayed produced aflatoxins B and G. In all, 24% of the 312 Texas *A. parasiticus*, 248 from soils and 64 from plant samples, were vegetatively compatible with one of the three Japanese sugarcane-associated *A. parasiticus* isolates. Fifteen isolates from sugarcane tissues were compatible with one of the sugarcane-associated *A. parasiticus* isolates. The VCG to which OPS500 belongs was the most common (47 RGV isolates), followed by the VCGs of OPS485 (22 RGV isolates) and OPS515 (3 RGV isolates).

Sequence of ITS regions (539 bp) contained only four parsimony-informative characteristics among 39 *A. flavus* and

A. parasiticus isolates. ML phylogenies from ITS sequence data showed three clades with >90% bootstrap support. All *A. flavus* isolates were restricted to a single clade, sister to the two clades that contained all the *A. parasiticus* isolates. *A. parasiticus* collected in association with sugarcane occupied a single clade, sister to *A. parasiticus* from culture collections, although sugarcane-associated *A. parasiticus* was only separated from culture collection *A. parasiticus* by one step and from *A. flavus* by four steps (data not shown).

Similar topologies were observed in the *aflR* (1.5 kb) and *niaD* (2.2 kb) trees generated from sequences that provided 66 and 153 parsimony-informative characteristics, respectively. The single most parsimonious MP phylogeny generated from the *aflR* sequence (Fig. 2) contained a bifurcated *A. parasiticus* clade (84%

Fig. 1. Percentage of *Aspergillus* section *Flavi* communities composed of *Aspergillus flavus*, *A. tamarii*, and *A. parasiticus* in Rio Grande Valley field soils with known sugarcane cropping histories. An average of 16 section *Flavi* isolates were collected from each soil. Levels of *A. flavus* (A, B, and C) and *A. parasiticus* (X, Y, and Z) without a common letter are significantly different by Fisher's protected least significant difference test ($P < 0.05$). Levels of *A. tamarii* were statistically similar for all treatments.

TABLE 3. *Aspergillus* section *Flavi* community from fields cropping and not cropping sugarcane in the Rio Grande Valley, Texas

Texas county	Sample date	Crop history ^x	Fields (N) ^y	Species composition (CFU) ^z		
				<i>Aspergillus flavus</i>	<i>A. parasiticus</i>	<i>A. tamarii</i>
Hidalgo	April 2004	Sugarcane	2	4 b	9 a	ND a
		No sugarcane	23	48 a	ND b	2 a
	April 2005	Sugarcane	4	4 b	15 a	1 a
		No sugarcane	17	61 a	ND b	2 a
	June 2005	Sugarcane	14	30 a	9 a	1 a
		No sugarcane	2	51 a	ND b	4 a
Cameron	June 2005	Sugarcane	11	36 a	26 a	5 a
		No sugarcane	6	69 a	0.8 b	2 a
		Sugarcane total	31	27 B	16 A	3 A
		No sugarcane total	48	55 A	0.1 B	2 A

^x Sugarcane = field cropped to sugarcane at least once within the previous 5 years and No sugarcane = field not cropped to sugarcane during the previous 5 years.

^y Number of fields were sampled by collecting one to four 200-g soil samples composed of 30 to 40 subsamples taken from the top 2 cm of soil along a diagonal transect of 80 to 100 m.

^z CFU per gram of soil determined with dilution plate technique. Limit of detection was 1 CFU g⁻¹. For each paired comparison, values not followed by the same letter in each column are significantly different with a two-sided Student's *t* test ($P < 0.05$). Statistical testing was performed on log + 1 transformations of the CFU data. ND = not detected.

bootstrap support), sister to *A. flavus*. The *A. parasiticus* clade showed two highly supported lineages: one including only sugarcane-associated isolates and the other including only isolates collected from non-sugarcane sources, which included the mealybug-isolated lectotype. These lineages were also distinct and well supported in ML analyses, with 98 and 99% bootstrap support for the non-sugarcane- and sugarcane-associated clades, respectively. The MP phylogeny created from the *niaD* sequence (Fig. 3) maintained the separation between the clade containing *A. flavus* (100% bootstrap support) and that containing *A. parasiticus* (98% bootstrap support). All isolates associated with sugarcane again formed a strongly supported clade (97% bootstrap support); however, this clade groups within a polytomy containing the *A. parasiticus* isolates from crops other than sugarcane. ML phylogenies also separated *A. parasiticus* and *A. flavus* species into highly supported clades (>90% bootstrap support), and bifurcated *A. parasiticus* into sister clades separating isolates from sugarcane and those from other sources (>88% bootstrap support; data not shown).

DISCUSSION

This is the first report to show that sugarcane specifically influences presence and persistence of one lineage of aflatoxin-producing fungi in RGV. *A. parasiticus* is almost exclusively associated with sugarcane in RGV, occurring primarily on sugarcane stems and in field soils with a recent history of sugarcane cropping. *A. parasiticus* from RGV sugarcane form a distinct phylogenetic lineage with Japanese isolates to the exclusion of *A. parasiticus* from other crops. The evolutionary divergence of *A. parasiticus* found in association with sugarcane from the rest of the species, even the ex-type isolate (NRRL 502) from a sugarcane mealybug (77), suggests that the association between *A. parasiticus* and sugarcane is not ephemeral. Vegetative compatibility analyses indicate that *A. parasiticus* VCGs associated with sugarcane in Japan are common among the RGV *A. parasiticus* isolates from both sugarcane tissues and soils planted to sugarcane. Although the physiological basis for the observed sugarcane-*A. parasiticus* relationship is unknown, *A. parasiticus* dominates the *Aspergillus* section *Flavi* community resident on RGV sugarcane and, in so doing, influences the aflatoxin-producing potential of that community and the etiology of aflatoxin contamination of both sugarcane products and rotation crops (49,74). Thus, associations of *A. parasiticus* with specific hosts impact the epidemiology of aflatoxin contamination by influencing the average aflatoxin-producing potential of fungal communities. Increased aflatoxin-producing potential has been associated with the most lethal aflatoxin contamination events globally

(73,74), and atoxigenic *A. parasiticus* are rare (22,76,88). Aflatoxin-producing fungi are saprophytes and facultative pathogens with broad host ranges, including plants and animals (16,36,78). Individual isolates infect and decay hosts that diverged millions of years before the present (35). However, a broad potential host range does not eliminate the possibility of host preference or competitive advantage among aflatoxin-producing fungi (63).

Sugarcane cultivation is frequently accompanied by specific lineages of *A. parasiticus* which require host cultivation for persistence. *A. parasiticus* was dominant in RGV fields rotated out of sugarcane for 1 year but undetectable in soil *Aspergillus* section *Flavi* communities after 5 years (Fig. 1; Table 4). *A. parasiticus* has most frequently been associated with peanut in the United States (39,41), and previous reports correlate the presence of *A. parasiticus* with soils suitable for peanut cultivation. However, peanut production has not been shown to increase proportions of *A. parasiticus* within soil fungal communities and rotation out of peanut has not been associated with loss of *A. parasiticus* (41). *A. parasiticus* is found in the peanut-growing regions of the southern United States but, elsewhere, peanut crops are not always associated with *A. parasiticus* (50,59,76,86) and, in some regions, *A. flavus* and not *A. parasiticus* is the primary causal agent of aflatoxin contamination of peanut (26,58,85). *A. parasiticus* isolates found in RGV were more closely related to *A. parasiticus* associated with sugarcane production in Japan than to U.S. isolates not associated with sugarcane. Spore banks have been implicated as the source of primary inoculum for *A. parasiticus* isolates infecting crops (30,32,38,44,76). *A. parasiticus* has been described as the member of *Aspergillus* section *Flavi* best adapted to the soil habitat (39–41). Sugarcane-associated *A. parasiticus* isolates are divergent from the rest of the species and, based on the response of these aflatoxin-producers to sugarcane production, occupy a distinct ecological niche. The *A. parasiticus* lectotype (NRRL502) is more closely related to those *A. parasiticus* isolates from maize and peanut than *A. parasiticus* found in association with sugarcane in RGV, although the lectotype was isolated from a sugarcane mealybug. Although peanut-associated *A. parasiticus* does not appear to be as host-dependent as sugarcane-associated *A. parasiticus*, host associations may drive species and population level divergence (19,64,79).

A. parasiticus isolates found on sugarcane in RGV were as phylogenetically distinct from *A. parasiticus* isolated from other crops as both groups are from *A. flavus*. In phylogenies based on *afIR* sequence, sugarcane-associated *A. parasiticus* form a sister clade to other *A. parasiticus* isolates, while *niaD* phylogenies nest sugarcane-associated *A. parasiticus* in a clade shared with *A. parasiticus* from other sources. Morphological resemblance of the

TABLE 4. Composition of *Aspergillus* section *Flavi* community isolated from sugarcane billets and field-collected stem pieces in RGV 2005

Source, county ^w	N ^x	<i>Aspergillus parasiticus</i> ^y		<i>A. flavus</i> ^y		Total isolates ^y	<i>A. parasiticus</i> (%) ^z	<i>A. flavus</i> (%) ^z
		With	Without	With	Without			
Billet								
Cameron	19	19	0	1	18	175	99 (A)	1 (C)
Hidalgo	10	10	0	3	7	148	89 (A)	11 (B,C)
Willacy	1	1	0	0	1	13	100 (A)	ND (A,B,C)
Total	30	30 A	0 B	4 B	26 A	336	95 (A)	5 (B)
Field								
Cameron	24	11	13	20	4	108	29 (B)	71 (A)
Hidalgo	23	19	4	14	9	233	74 (A)	25 (B)
Total	47	30 B	17 A	34 A	13 B	341	52 (B)	48 (A)

^w Conventionally harvested billet samples provided by Rio Grande Valley Sugar Growers, Inc., sugar mill; field samples were hand-collected in June.

^x Number of individual plants sampled; 1 stem per sample.

^y Number of stems from which at least one *A. flavus* or *A. parasiticus* was isolated or from which no *A. flavus* or no *A. parasiticus* was isolated. Totals were subjected to statistical analyses. Comparisons frequencies of *A. parasiticus* and *A. flavus* were compared between the total field and billet samples. Numbers not followed by the same letter differ by the Pearson's χ^2 test; $\chi^2_{1,77} = 13.9$, $P = 0.0002$, for number of *A. parasiticus*-positive stems and $\chi^2_{1,77} = 25.5$, $P < 0.0001$, for number of *A. flavus*-positive stems.

^z Percentage of isolates belonging to either *A. flavus* or *A. parasiticus*. Percentages followed by the same letter are not significantly different ($P = 0.05$) by Fisher's protected least significant difference (LSD) test. Statistical tests utilized arcsine transformations of the percentage data. ND = not detected.

genetics of *A. parasiticus* associated with sugarcane to those associated with other crops (Figs. 2 and 3) invalidates the use of conidial shape and colony color for identification of divergent adaptations and distributions among lineages within *Aspergillus* section *Flavi*. However, morphology of sclerotia and habits of sclerotia formation may provide useful characteristics for differentiating *A. parasiticus* clades, as is the case for other species within *Aspergillus* section *Flavi* (10,28,30). Molecular markers specific to each lineage would facilitate investigations on lineage specific characteristics, and the sequence data developed for *niaD* and *aflR* during the current study may be an adequate basis for such markers, whereas *ITS* sequence was uninformative.

A. parasiticus was previously associated with aflatoxins in raw sugar but frequencies and severities of contamination in sugar products are unreported (84) because procedures for refining sugar from sugarcane do not concentrate aflatoxins in either sugar or valuable byproducts. Concentration of aflatoxins does occur during production of both high-protein meals in vegetable oil

production and dry distiller grains in ethanol production (14,95). Indeed, high heat and calcium hydroxide clarification during table-sugar processing may degrade any aflatoxins present (6,21) but raw sugar products made without harsh treatments may be at higher risk of aflatoxin contamination (54,82). Despite the limited risk posed to sugar products, *A. parasiticus* causes severe contamination of staple crops, including peanut, produced in Africa and North America (16,20,39). The impact of sugarcane-associated *A. parasiticus* on aflatoxin contamination in other crops in the United States is unknown. Commercial crops in the RGV are frequently assayed for total aflatoxin content, and G aflatoxins, a metabolite of *A. parasiticus* but not *A. flavus*, are not quantified independently. However, two *A. parasiticus* isolates from cotton lint in the RVG were found to be of the type associated with sugarcane in the current study. High levels of persistence of sugarcane-associated *A. parasiticus* in field soils 1 year after rotation out of sugarcane provides potential for rotation crop exposure to these aflatoxin-producers.

A. parasiticus isolates dominated the *Aspergillus* section *Flavi* community in stem pieces (billets) prepared for planting and were

Fig. 2. *Aspergillus* section *Flavi* phylogeny inferred by maximum parsimony from 1.5 kb of *aflR*, the gene encoding the transcription factor that regulates many aflatoxin biosynthesis genes. The single most parsimonious tree is shown, with nodes of <80% bootstrap support collapsed. Maximum-likelihood analysis resulted in similar topology, with >90% bootstrap support for the clades separating *Aspergillus flavus*, sugarcane-associated *A. parasiticus*, and *A. parasiticus* from other sources. Numbers above branches are branch lengths proportional to the number of character changes and those below are bootstrap frequencies based on 1,000 replicates. Labels on the phylogeny are either culture collection names or laboratory working names. The tree is rooted with *A. nomius* (NRRL 13137) as the outgroup. All *A. parasiticus* isolates associated with sugarcane cropping are included in the dashed box.

Fig. 3. *Aspergillus* section *Flavi* phylogeny inferred by maximum parsimony from 2.2 kb of the nitrate reductase gene *niaD*. The single most parsimonious tree is shown, with nodes of <80% bootstrap support collapsed. Maximum-likelihood analysis resulted in similar topology, with 91% support for the *Aspergillus flavus* clade and 99% for the *A. parasiticus* clade. Sugarcane-associated *A. parasiticus* isolates were in a strongly supported clade (88% bootstrap support), while *A. parasiticus* from other sources formed a clade not resolved on this tree. Numbers above branches are branch lengths proportional to the number of character changes along the branch and those below are bootstrap frequencies based on 1,000 replicates. Labels on the phylogeny are either culture collection names or laboratory working names. The tree is rooted with *A. nomius* (NRRL 13137) as outgroup. All *A. parasiticus* isolates associated with sugarcane cropping are included in the dashed box.

absent from fields with no history of sugarcane cropping. This clearly demonstrates that sugarcane billets can be vehicles by which *A. parasiticus* genotypes move within agricultural areas (Table 4). The *A. parasiticus* lineage associated with RGV sugarcane in the current study was previously described in Japan, also on sugarcane. In agroecosystems, *A. parasiticus* populations differ from the contiguous, widespread, and genotypically diverse populations of *A. flavus* (7), in that *A. parasiticus* populations are discrete, often associated with a limited number of crop species, and composed of relatively fewer genotypes (8,40,41,58). Diverse VCGs coexist in *A. parasiticus* populations, although particular VCGs may be rare in certain regions. The VCGs of each of the three *A. parasiticus* isolates from sugarcane cultivated in Japan were found in RGV, with 24% of >300 tested RGV *A. parasiticus* isolates belonging to one of these VCGs. In *Aspergilli*, membership in a VCG indicates close relatedness to other individuals within the same VCG (4,34,57). It is common for a VCG to be distributed across long distances, although previous reports have shown this only across contiguous agricultural areas (41,42). The current report is the first to document distribution of *A. parasiticus* VCGs between widely separated continents. Sugarcane cultivation may have influenced movement of these VCGs between Asia and North America. Sugarcane breeding programs rely on global germplasm collections, and U.S. programs often include Japanese accessions (67).

The association of *A. parasiticus* with sugarcane (Fig. 1; Table 3) observed in the current study resulted in sugarcane cropping changing the structure of *Aspergillus* section *Flavi* soil communities. These changes to the fungal community were more readily detected in the RGV because *A. parasiticus* does not persist long in this region in the absence of sugarcane. The lack of persistence results in *A. parasiticus* composing very low proportions of *Aspergillus* section *Flavi* communities when crops other than sugarcane are grown. Other crop rotations also alter structures of communities of aflatoxin-producing fungi (34,48,49,76). Crop rotation effects on *A. parasiticus* may have been obscured in studies examining relationships between *A. parasiticus* and peanut production by either pooling results from 3 years (1), examining influences of crop rotations over only a single season (33,43), or analyzing soils from fields with unknown cropping histories and mixed cropping regimes (37). *A. parasiticus* lineages associated with sugarcane are apparently dependent on that crop in the RGV. This dependence may reflect lack of competitiveness of these non-native fungi with RGV microflora and in the RGV soil environment. Similarly, non-native *A. parasiticus* isolates of peanut origin did not persist when applied to Arizona peanut fields (94). The extent to which *Aspergillus* section *Flavi* communities shift during crop rotation is dependent on adaptations of the constituent fungi to the soil environment, including soil chemistry, crop debris, and soil water potential (46). Processes by which specific crops favor one species, lineage, or genotype within *Aspergillus* section *Flavi* have been largely unexplored. Shifts in community compositions following rotation of sugarcane to other crops may be attributable to both plant-created environments that favor *A. parasiticus* and competitive differences among lineages within *Aspergillus* section *Flavi* that may be favored by other crops (15,63).

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