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Sexual reproduction and recombination in the aflatoxin-producing fungus *Aspergillus parasiticus*

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ABSTRACT

The fungal phylum Ascomycota comprises a large proportion of species with no known sexual stage, despite high genetic variability in field populations. One such asexual species, *Aspergillus parasiticus*, is a potent producer of carcinogenic and hepatotoxic aflatoxins, polyketide-derived secondary metabolites that contaminate a wide variety of agricultural crops. In this study, individuals of *A. parasiticus* from a population showing an evolutionary history of recombination were examined for sexual reproduction. Crosses between strains with opposite mating-type genes *MAT1-1* and *MAT1-2* resulted in the development of ascospore-bearing ascocarps embedded within stromata. Sexually compatible strains belonged to different vegetative compatibility groups. Recombination through the independent assortment of chromosomes 3 and 6 was detected using loci for mating type, aflatoxin gene cluster, and a protein-encoding gene. Our discovery of the sexual stage in *A. parasiticus* has important implications for current biological control strategies using nontoxigenic strains to reduce aflatoxin contamination in crops.

1. Introduction

Aflatoxins are among the most potent carcinogens known and also exhibit acute hepatotoxic and immunosuppressive properties (Eaton and Groopman, 1994; Turner et al., 2003). These polyketide-derived secondary metabolites are produced by fungi in agricultural commodities, notably corn, peanuts, tree nuts, cottonseed, and spices (Payne, 1998). Outbreaks of human aflatoxicosis occur in regions where highly contaminated food is ingested, most recently in Kenya, East Africa (Azziz-Baumgartner et al., 2005), and over 100 countries have stringent regulatory limits on allowable concentrations in human and animal foods (van Edmond and Jonker, 2005). The two fungal species responsible for aflatoxin contamination, *Aspergillus flavus* and the closely related *A. parasiticus*, reproduce clonally by means of asexual spores and have no previously known sexual stage, despite high variability in morphology, toxigenicity, and genetic characters in field populations (Horn, 2007). *Aspergillus flavus* and *A. parasiticus* belong to a vast assemblage of asexual species comprising approximately 40% of the fungal phylum Ascomycota (Reynolds, 1993). Nearly every asexual fungal species that has been examined at the molecular level, including the potential human pathogens *Aspergillus fumigatus* (Paoletti et al., 2005) and *Coccidioides immites* (Burt et al., 1996), shows evidence of recombination, bringing to question as to how many truly clonal species exist in nature.

Molecular characterization of *A. flavus* and *A. parasiticus* in recent years has suggested the existence of an undiscovered sexual stage, despite many decades of unsuccessful attempts to induce sexuality. Under the model of clonality, gene genealogies inferred from multiple loci are concordant, since the genes are inherited together without recombination associated with sex. However, gene genealogies inferred from nuclear loci in *A. flavus* differ markedly in their topologies, indicating recombination and possible sexual reproduction (Geiser et al., 1998). Aflatoxin biosynthesis involves approximately 25 enzymes encoded by genes that are clustered together in a 70-kb telomeric region on chromosome 3 (Carbone et al., 2007b; Yu et al., 2004). Four structurally related aflatoxins occur in nature, depending upon the presence of polyketide dihydro- (B_1 and G_1) or tetrahydro- (B_2 and G_2) bisfuran rings. Of these aflatoxins, B_1 is the most toxic and carcinogenic (Eaton and Groopman, 1994). We previously reported that the aflatoxin gene cluster in *A. parasiticus* shows significant linkage disequilibrium over the evolutionary history of the species and that the cluster can be partitioned into five distinct recombination blocks (Carbone et al., 2007a). Furthermore, these analyses showed evidence of contemporary recombination.

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Sexual compatibility in ascomycetous fungi is determined by structurally unrelated *MAT1-1 and MAT1-2* genes that are located at a single locus (Debuchy and Turgeon, 2006). *MAT1-1* encodes for a protein with an α-box motif and *MAT1-2* encodes for a protein of the high mobility group (HMG). An individual with only one of the genes requires the opposite mating type for sexual reproduction (heterothallism). We recently showed that *A. flavus* and *A. parasiticus* strains contain a single *MAT1-1* or *MAT1-2* mating-type gene and therefore the species are heterothallic (Ramirez-Prado et al., 2008). Gene expression at the mRNA level by *MAT1-1* and *MAT1-2* also was detected in growing mycelia. Furthermore, our examination of *A. flavus* and *A. parasiticus* populations from a peanut field in Georgia, USA, showed that clone-corrected populations of both species comprise an approximate 1:1 ratio of mating types (Ramirez-Prado et al., 2008). In summary, recombination within the aflatoxin gene cluster, expression of *MAT* genes at the mRNA level, and an equal distribution of mating types in populations together provide strong evidence for sexual reproduction by aflatoxigenic fungi in nature. Individuals within *A. flavus* and *A. parasiticus* populations vary widely in their ability to produce aflatoxins, ranging from those that are nonaflatoxigenic to those that are potent producers of aflatoxins (Horn and Dorner, 1999). Sexual recombination could account for much of this natural variation in toxicity.

In the present research, individuals of *A. parasiticus* from a population showing an evolutionary history of recombination (Carbone et al., 2007a) were examined for sexual reproduction. Crosses between strains with opposite mating-type genes *MAT1-1* and *MAT1-2* resulted in the development of ascospore-bearing ascocarps embedded within stromata. Progeny from these crosses were analyzed for evidence of genetic recombination.

2. Materials and methods

2.1. Culture conditions

Strains of *A. parasiticus* were obtained from soil and peanut seeds in a peanut field in Terrell County, Georgia, USA, and have been characterized according to morphology, vegetative compatibility, and aflatoxin production (Horn and Greene, 1995; Horn et al., 1996). In previous research, we grouped these strains into haplotypes (alleles) based on sequence variation in 21 intergenic regions of the aflatoxin gene cluster (Carbone et al., 2007a) and also identified their mating-type genes *MAT1-1* and *MAT1-2* (Ramirez-Prado et al., 2008). Fungal strains were initially grown on Czapek agar (Cz) (Raper and Fennell, 1965) slants for 14 days at 30 °C. Dry conidia from these cultures were suspended as either single strains or paired strains of the opposite mating type in 0.2% (w/v) water agar containing 50 μL/L Tween 20 (approximately 5×10^5 conidia/mL of each strain). Conidial suspensions (7 μL) were spread onto each of five slants of mixed cereal agar (McAlpin and Wicklow, 2005) (7 mL medium in 20 × 125-mm screw-cap test tubes), and slants were incubated in darkness for 14 days at 30 °C to induce sclerotium production. Slant cultures (caps loose) were then enclosed in sealed plastic bags to prevent desiccation and incubated an additional 6–9 months at 30 °C.

2.2. Dissections of sclerotia and stromata

Sclerotia/stromata were harvested by adding 10 mL water with Tween 20 to each culture slant, scraping the agar surface with a transfer loop, and filtering the suspension through a 100-mesh filter. The retained sclerotia/stromata were transferred to a vial, repeatedly vortexed in water followed by decanting to remove residual conidia, and filtered onto Whatman #4 filter paper. Stromata selected for obtaining single-ascospore cultures were further cleaned by vortexing 1–2 min in 10 mL sterile water containing 1 g glass beads (200–350 μm diameter), filtering to remove from the beads, and vortexing and decanting twice with water. Sclerotia/stromata were dissected with a microscalpel and examined under the stereomicroscope.

2.3. Single-ascospore isolates

The contents of a single ascocarp were removed from each stroma with a microneedle and suspended in vials containing 1 mL sterile 0.1 M potassium phosphate buffer (pH 7). Vials were then immersed in a water bath for 3 min at 50 °C to kill a portion of the vegetative hyphae and other non-ascospore elements. Aliquots of the suspensions (0.1 mL) were spread on each of five plates of malt extract agar (Raper and Fennell, 1965), and plates were incubated for 20–25 h at 30 °C. Germlings were identified under the light microscope (200×) by the presence of an ascospore at the origin point and often by the presence of a large oil droplet, which remained within the ascospore or migrated varying distances down the germ tube. Ascospores were easily distinguished from the rarely observed conidia by their thick, nearly smooth walls and relatively large size (9.7 × 9.0 μm), in contrast to conidia, which are thinner walled, coarsely roughened, and smaller (4–6 μm). Individual germlings were transferred to Cz plates and grown 3–5 days at 30 °C. Hyphal tips from the colony margins were then transferred to Cz slants.

2.4. Light microscopy

Low-magnification photographs of entire sclerotia/stromata were obtained with a Leica MZ16FA stereomicroscope equipped with a DFC-300FX digital camera. Stacked images were combined using Image Pro Express v.5.0 software module. Medium- and high-magnification photographs of asci and ascospores were taken with a Nikon CoolPix 995 digital camera mounted on a Nikon Eclipse E600 compound microscope equipped for differential interference contrast.

2.5. Sample fixation for scanning electron microscopy

Hand-sectioned stromata were fixed for at least 24 h in 3% glutaraldehyde (EM grade)/0.1 M sodium cacodylate buffer (pH 7.3) at 4 °C, then transferred by pipette to 120–200 μm SPI microporous capsules (SPI Supplies, West Chester, PA) containing cold sodium cacodylate buffer. Samples remained in the capsules for all subsequent solution changes. The volumes used were at least 10× the volume of the sample to minimize changes in concentration. Samples were rinsed with occasional agitation in three 1-h changes of cold 0.1 M sodium cacodylate buffer (pH 7.3) followed by 1-h changes of cold 30% ethanol and 50% ethanol. Samples were left in 70% ethanol overnight at 4 °C followed by an 8-h change of cold 95% ethanol with occasional swirling. After this, the samples were placed in cold 100% ethanol and allowed to come to room temperature for 24 h. Two more 24-h changes of 100% ethanol at room temperature completed the dehydration sequence.

2.6. Scanning electron microscopy

Samples were critical-point dried in liquid carbon dioxide using a Samdri-795 Critical-Point Drier (Tousimis Research Corp., Rockville, MD) according to the manufacturer's directions. Pressure and temperature were held constant for 15 min at the critical point. Samples were removed from the chamber and were either mounted on stubs or left unmounted, then stored in a desiccator under vacuum. For mounting specimens, SEM sample holders were prepared with double-stick tape and sectioned stromata were