ABSTRACT

GELL, RICHARD MICHAEL. Consequences of Sexual Reproduction in *Aspergillus flavus:* Development of Approaches for the Examination of F1 Progeny. (Under the direction of Dr. Ignazio Carbone).

The production of carcinogenic aflatoxins by the fungus *Aspergillus flavus* on agricultural commodities causes health and economic consequences worldwide. Current management practices utilize the high-density application of commercial non-aflatoxin producing fungal strains, primarily Afla-Guard® and AF36 within the United States, to act as biocontrols and eliminate the detection of relevant quantities of aflatoxin. The recent discovery of the sexual cycle in *A. flavus*, with the production of viable F1 offspring, resulted in many questions about the role of genetic inheritance in this highly variable filamentous fungus.

In addition to controlled laboratory crosses, we show *A. flavus* sclerotia, survival structures composed of hardened and melanized mycelia, are capable of undergoing the mating process with wild populations both in the lab and field indicated by the presence of alleles from the known parent as well as novel alleles in harvested progeny strains. This also led to the initial indication of an anisogamous reproduction system, where the two parental gametes differ functionally, through examination of reciprocal crosses between sclerotia and conidia of paired strains due to the inheritance of mitochondrial DNA from only the sclerotial parent but inheritance of nuclear loci from both parental strains. A drastic difference in the production of fertile sclerotia depending on which strain is provided as sclerotia within the reciprocal crosses and the inclusion of both major non-aflatoxin producing biocontrol strains among the parental strains utilized presented the opportunity to begin to genetically map these variable phenotypic traits in *A. flavus*.

The ability to reliably phenotype large numbers of progeny strains for a selected trait was discovered to be an immediate concern for linkage mapping of genes. Of greatest interest is the variation these progeny strains may contain, and the underlying genetic origins, in their production of aflatoxin. The methodologies for aflatoxin extraction and quantification from culture using chromatography exist, but no approach was sufficiently small scale to handle the number of cultures necessary for progeny panels or large population surveys while retaining the necessary reliability. We developed and provide details of a method for preparation and quantitation of aflatoxin B_1, the most abundantly produced form of aflatoxin, from fungal cultures which satisfies those needs, and show its utility through the initial examination of over forty strains collected from natural populations.

An additional necessary piece towards linkage mapping is the construction of the first genetic maps of *A. flavus*. These maps are based on three mapping populations each composed of the parental strains and approximately 70 F1 progeny. Genome wide data using double digest Restriction Associated DNA sequencing identified 496, 811, and 576 significant polymorphisms differentiating parents across eight linkage groups to serve as markers. These markers allowed for the production of genetic maps with total lengths of 1504.4, 1669.2, and 2001.3 cM. Recombination events were non-randomly distributed across chromosomes with an average rate of recombination of 46.81 cM per Mbp. We further support the discovery of anisogamous inheritance of mitochondrial loci from the sclerotial strain in crosses, whereas nuclear loci show a 1:1 segregation ratio from both parents. These genetic maps give the first insights into the potential for genetic exchange within *A. flavus* after only one generation.

These understandings of inheritance in the lab and within controlled field crosses provided the tools necessary for interpreting the results from population samples. Natural field

populations were compared to populations three months and one year after application of a biocontrol strain from fields across four states. *A. flavus* strains from the post-biocontrol application populations contained genomic regions associated with the biocontrol. These strains at three months out show evidence of recombination in the field at rates consistent with a single generation. The strains at a year out begin to show the long-term effects of the sexual cycle and recombination with the introgression of genes from the biocontrols into the population.

© Copyright 2019 by Richard Michael Gell

All Rights Reserved

Consequences of Sexual Reproduction in *Aspergillus flavus:* Development of Approaches for the Examination of F1 Progeny.

by
Richard Michael Gell

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Genetics

Raleigh, North Carolina
2019

APPROVED BY:

Dr. Ignazio Carbone Committee Chair	Dr. David Aylor
Dr. Marc Cubeta	Dr. Peter Ojiambo

DEDICATION

To my parents, Terry and Susan Gell, who always knew I could succeed.

BIOGRAPHY

Richard Michael Gell was born on May 15th, 1989, in Cincinnati, Ohio. In 2011, he graduated from the University of Georgia with his Bachelor of Science in Biology and Psychology. While attending university and for a year afterwards, he began research in the lab of Dr. R. Kelly Dawe under the guidance of Dr. Jonathan Gent studying small interfering RNAs originating from centromere associated repetitive elements in *Zea mays*. In the summer of 2012, Richard moved to Raleigh, North Carolina to attend graduate school at North Carolina State University in the Department of Genetics. He worked with Drs. Jose Alonso and Anna Stepanova on auxin synthesis and cell communication in *Arabidopsis thaliana* before joining the lab of Dr. Ignazio Carbone where he studies the sexual cycle and its impact through genetic inheritance in *Aspergillus flavus*.

TABLE OF CONTENTS

LIST OF TABLES vi
LIST OF FIGURES vii

Chapter 1: Overview 1
References 4

Chapter 2: Sexual Reproduction in *Aspergillus flavus* Sclerotia: Acquisition of Novel Alleles from Soil Populations and Uniparental Mitochondrial Inheritance 6
Contribution Summary 7
Abstract 8
Introduction 9
Materials and Methods 12
  Fungal strains and sclerotium productions 12
  Laboratory incubation of single-strain sclerotia on soil 13
  Field incubation of single-strain and fertilized sclerotia 14
  Reciprocal crosses between single-strain sclerotia and conidia 16
  Mating-type determination and genotype analyses 16
Results 19
  Laboratory incubation of single-strain sclerotia on soil 19
  Field incubation of single-strain and fertilized sclerotia 20
  Reciprocal crosses and mitochondrial inheritance 22
Discussion 24
References 30
Supplemental Text 53

Chapter 3: HPLC Quantitation of Aflatoxin B1 from Fungal Mycelium Culture 56
Contribution Summary 57
Abstract 58
Introduction 59
Materials and Methods 61
  Solid and Liquid Fungal Cultures for Aflatoxin Analysis 61
  Aflatoxin Extractions 61
  Extract Purification 62
  HPLC 62
  Aflatoxin Recovery from Spiking 63
  Known Aflatoxigenic Strains and Population Sample Isolates 64
Results and Discussion 65
  Aflatoxin Recovery from Spiking 65
  Aflatoxin Recovery from Known Aflatoxigenic Strains 66
  Aflatoxin Recovery from Population Sample Isolates 66
Conclusions 68
References 69

Chapter 4: Genetic Map and Heritability of *Aspergillus flavus* 79
Contribution Summary 80
Abstract 81
Introduction 82

Materials and Methods 85
Fungal Strains 85
Mitochondrial Variants 85
DNA Sequencing 86
Genotype Calling and Mapping 86
Recombination Fractions and Determining Linkage 87
Recombination Rate 88
Results 90
Mitochondrial Calls 90
Progeny Statistics 90
Recombination Fractions and Determining Linkage 92
Genetic Maps 93
Recombination Rate 94
Discussion 95
References 99

LIST OF TABLES

Chapter 2

Table 2.1 Sexual reproduction in *A. flavus* sclerotia from single strains of one mating type when incubated on sterile soil and on soil containing natural fungal populations. . 35
Table 2.2 Sexual reproduction in single-strain and fertilized sclerotia of *A. flavus* under field conditions. 36
Table 2.3 Reciprocal crosses in *A. flavus* in which single-strain sclerotia were incubated on sterilized soil inoculated with conidia. 38
Table 2.4 Sexual reproduction in *A. flavus* sclerotia obtained from crosses and incubated under laboratory conditions. 39
Table 2.5 Genotype data for *A. flavus* progeny from single-strain sclerotia fertilized by strains from natural soil populations when incubated under laboratory conditions. 40
Table 2.6 Soil populations of *Aspergillus* section *Flavi* species (CFU/g) in fields prior to application of *A. flavus* sclerotia. 42
Table 2.7 Genotype data for *A. flavus* progeny from single-strain sclerotia fertilized by strains from natural soil populations in the field. 43
Table 2.8 Genotype data for *A. flavus* progeny obtained from fertilized sclerotia that were applied to fields before ascocarp formation. 45
Table 2.9 Nuclear and mitochondrial loci for progeny from reciprocal crosses between single-strain sclerotia and conidia inoculated in sterile soil. 49
Table 2.S1 Weather conditions at three fields (2013-2014) where single-strain and fertilized sclerotia of *A. flavus* were applied. 52

Chapter 3

Table 3.1 Aflatoxin concentrations and recovery in experimental and spiked conditions. 72
Table 3.2 Aflatoxin B_1 concentrations from liquid culture of known aflatoxigenic strains. 73
Table 3.S1 Aflatoxin B_1 concentrations from liquid culture of *A. flavus* population sample isolates by genetic lineage. 74

Chapter 4

Table 4.1 Initial and filtered progeny individuals and markers 103
Table 4.2 Initial genetic map lengths in centimorgans for the complete map and each chromosome based on *A. oryzae* RIB40 104
Table 4.3 Comparison of genetic lengths with *A. oryzae* RIB40 marker organization and the new organization for chromosomes 2 and 6 105

LIST OF FIGURES

Chapter 3

Figure 3.S1 Aflatoxin typical standard chromatogram with aflatoxin B_1 at 0.5 μg/mL, B_2 at 0.25 μg/mL, G_1 at 0.5 μg/mL, and G_2 at 0.05 μg/mL. 78

Chapter 4

Figure 4.1 Parental genotype frequencies of progeny strains.. 106
Figure 4.2 Comparison of pairwise recombination fractions and logarithm of the odds (LOD) scores for Chromosome 2 and Chromosome 6 of each cross 109
Figure 4.3 Genetic maps of chromosomes involved in inverted reciprocal translocation 111
Figure 4.4 Genetic maps showing marker distribution 112
Figure 4.5 Recombination rate variation across the genome for each cross 115
Figure 4.S1 Pairwise recombination fractions and LOD scores for each cross......................... 117
Figure 4.S2 Genetic maps of chromosomes involved in inverted translocation 121

CHAPTER 1

Overview

Aflatoxins (AFs) contamination is a persistent concern for growers of many agricultural staples around the world (Grace *et al.* 2015; Khlangwiset & Wu 2010). Produced by *Aspergillus flavus*, consumption of these potent mycotoxins compromises critical components of health and are strongly hepatotoxic, frequently causing liver cancer (Kew 2013; Williams *et al.* 2004). Current control methods involve high-density application of non-toxin producing strains of *A. flavus* as a biological control strain (Dorner 2004). The two biocontrol agents Afla-Guard® and AF36 regularly used in regions of the United States were selected due to mutations in critical pathway genes, and biocontrol agent development continues to focus on this trait (EPA 2004; Tran-Dinh *et al.* 2014).

Production of AFs by *A. flavus* relies on both environmental and genetic factors. Environmental sources of nitrogen and carbon, water activity as well as pH levels all influence the ability of *A. flavus* to produce AFs (Gallo *et al.* 2016; Woloshuk & Shim 2013). Temperature is another environmental factor that strongly regulates AF production with maximal production occurring between 28°C and 30°C and decreases as the temperature approaches 37°C or 20°C (Gallo *et al.* 2016; O'Brian *et al.* 2007; Schindler *et al.* 1967). This decrease in AF production due to temperature corresponds to decreased expression of many AF biosynthetic genes (O'Brian *et al.* 2007). The majority of AF biosynthetic genes are located in the AF gene cluster, a 70-kb region in the subtelomeric region of chromosome 3 (Yu *et al.* 1995). AF cluster genes, including *aflR* and *aflJ,* were the first described regulators of AF biosynthesis, but recent discoveries point towards an increasing influence of genome-wide regulation as playing an important role (Du *et al.* 2007; Payne *et al.* 1993). Environmental response genes such as *areA*

on Chromosome 1*,* which produces a nitrogen regulatory protein, *creA* on Chromosome 3*,* which is involved in carbon catabolite repression, and *pacC* on Chromosome 2*,* which responds to increasing pH are all associated with altered levels of AF biosynthesis (Price *et al.* 2005). Genes such as *laeA* and *veA* from Chromosome 2*,* which are components of the *velvet* nuclear complex, are involved in systemic secondary metabolite production; removal of either gene halts a strains ability to produce AF (Bok & Keller 2004; Duran *et al.* 2007; Kale *et al.* 2008).

In addition to the well-characterized asexual cycle, we recently described sexual reproduction in *Aspergillus flavus* in controlled laboratory conditions (Horn *et al.* 2009). When undergoing the sexual cycle, undefined cell types capable of forming from conidia of two strains of opposite mating types are capable of fusing in heterothallic mating (Horn *et al.* 2009; Ramirez-Prado *et al.* 2008). After incubation, fertilized sclerotia can be found to bear ascocarps containing the ascospore progeny. This mating reaction occurs between individuals from different vegetative compatibility groups (VCGs), which are incapable of fusion during the asexual cycle due to carrying different heterokayron incompatibility (*het)* loci (Leslie 1993). A VCG is a group of strains, which share alleles at all *het* loci allowing for hyphal fusion (anastomosis). This makes genetic exchange possible within a VCG via the parasexual cycle (Papa 1973). Despite the simpler mechanism, genetic exchange via the parasexual cycle has little influence on variation in a population's morphology and chemotype due to the majority of that variation occurring between VCGs (Horn *et al.* 1996). The sexual cycle generates novel toxin phenotypes and is capable of disseminating them through the population though greater understanding is needed on rates of fertility and recombination in *A. flavus* (Moore *et al.* 2013). The sexual cycle occurs though an interaction which requires the production of mature sclerotia (Horn *et al.* 2009). The fertility of strains is highly variable with some strains showing a

capability to mate with multiple strains from other VCGs with many sclerotia showing viable progeny. While other strains have produced limited fertile sclerotia with only one strain from another VCG or have failed to undergo the sexual cycle at all. The variation in the fertility of parental strains can be examined in their progeny to help identify factors, such as sclerotial development, and genes, such as *laeA* and *veA* that control production of both AFs and sclerotia, that may play a role.

Within this thesis, the role played by the sexual cycle in *A. flavus* continues to be examined. Chapter 2 shows the capability of strains to undergo the sexual cycle in natural environments and with natural populations as well as uncovering the first suggestion of an anisogamous reproductive system, where only one parental strain in each cross contributes mitochondria. Chapter 3 presents the means to determine aflatoxin concentrations for the sample sizes required when examining the role of non-aflatoxin cluster loci in heritability of aflatoxin production. Chapter 4 expands on the understanding of inheritance after one generation discussed above and in Chapter 2 through the development of the first genetic maps of *A. flavus* crosses, descriptions of the effects of recombination after one generation including recombination rates, and further supporting the discovery of anisogamous reproduction in *A. flavus*. Chapter 5 utilizes methodologies for determining aflatoxin presented in Chapter 3 and understandings of inheritance found in Chapter 4 to explore and understand results found in population isolates of *A. flavus* regarding co-existence of genomic regions associated with both biocontrol strains and native strains in samples harvested three months and a year after application of the biocontrol strain. These chapters work together to expand our understanding of the impact the sexual cycle has on the genetic landscape of *A. flavus* and serve as the foundation for future work on heritability of critical phenotypes.

REFERENCES

Bok JW, Keller NP (2004) LaeA, a regulator of secondary metabolism in Aspergillus spp. *Eukaryot Cell* **3**, 527-535.

Dorner JW (2004) Biological Control of Aflatoxin Contamination of Crops. *Journal of Toxicology: Toxin Reviews* **23**, 425-450.

Du W, Obrian GR, Payne GA (2007) Function and regulation of aflJ in the accumulation of aflatoxin early pathway intermediate in Aspergillus flavus. *Food Addit Contam* **24**, 1043-1050.

Duran RM, Cary JW, Calvo AM (2007) Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by veA, a gene necessary for sclerotial formation. *Appl Microbiol Biotechnol* **73**, 1158-1168.

EPA (2004) *Aspergillus flavus* (NRRL 21882) (PC Code 006500) [electronic resource]. U.S. Environmental Protection Agency, Office of Pesticide Programs.

Gallo A, Solfrizzo M, Epifani F, Panzarini G, Perrone G (2016) Effect of temperature and water activity on gene expression and aflatoxin biosynthesis in *Aspergillus flavus* on almond medium. *Int J Food Microbiol* **217**, 162-169.

Grace D, Mahuku G, Hoffmann V*, et al.* (2015) International agricultural research to reduce food risks: case studies on aflatoxins. *Food Security* **7**, 569-582.

Horn BW, Dorner JW (1999) Regional differences in production of aflatoxin B_1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Appl Environ Microbiol* **65**, 1444-1449.

Horn BW, Greene RL, Sobolev VS*, et al.* (1996) Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus, A. parasiticus,* and *A. tamarii*. *Mycologia* **88**, 574–587.

Horn BW, Moore GG, Carbone I (2009) Sexual reproduction in *Aspergillus flavus*. *Mycologia* **101**, 423-429.

Kale SP, Milde L, Trapp MK*, et al.* (2008) Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. *Fungal Genet Biol* **45**, 1422-1429.

Kew MC (2013) Aflatoxins as a cause of hepatocellular carcinoma. *J Gastrointestin Liver Dis* **22**, 305-310.

Khlangwiset P, Wu F (2010) Costs and efficacy of public health interventions to reduce aflatoxin-induced human disease. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **27**, 998-1014.

Leslie JF (1993) Fungal vegetative compatibility. *Annu Rev Phytopathol* **31**, 127-150.

Moore GG, Elliott JL, Singh R*, et al.* (2013) Sexuality generates diversity in the aflatoxin gene cluster: evidence on a global scale. *PLoS Pathog* **9**, e1003574.

O'Brian GR, Georgianna DR, Wilkinson JR*, et al.* (2007) The effect of elevated temperature on gene transcription and aflatoxin biosynthesis. *Mycologia* **99**, 232-239.

Olarte RA, Horn BW, Dorner JW*, et al.* (2012) Effect of sexual recombination on population diversity in aflatoxin production by Aspergillus flavus and evidence for cryptic heterokaryosis. *Mol Ecol* **21**, 1453-1476.

Papa KE (1973) The parasexual cycle in *Aspergillus flavus*. *Mycologia* **65**, 1201-1205.

Payne GA, Nystrom GJ, Bhatnagar D, Cleveland TE, Woloshuk CP (1993) Cloning of the afl-2 gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. *Appl Environ Microbiol* **59**, 156-162.

Price MS, Conners SB, Tachdjian S, Kelly RM, Payne GA (2005) Aflatoxin conducive and non-conducive growth conditions reveal new gene associations with aflatoxin production. *Fungal Genet Biol* **42**, 506-518.

Ramirez-Prado JH, Moore GG, Horn BW, Carbone I (2008) Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genet Biol* **45**, 1292-1299.

Schindler AF, Palmer JG, Eisenberg WV (1967) Aflatoxin Production by *Aspergillus flavus* as Related to Various Temperatures. *Appl Microbiol* **15**, 1006-1009.

Tran-Dinh N, Pitt JI, Markwell PJ (2014) Selection of non-toxigenic strains of *Aspergillus flavus* for biocontrol of aflatoxins in maize in Thailand. *Biocontrol Science and Technology* **24**, 652-661.

Williams JH, Phillips TD, Jolly PE*, et al.* (2004) Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr* **80**, 1106-1122.

Woloshuk CP, Shim WB (2013) Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge. *FEMS Microbiol Rev* **37**, 94-109.

Yu J, Chang PK, Cary JW*, et al.* (1995) Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. *Appl Environ Microbiol* **61**, 2365-2371.

CHAPTER 2

Sexual Reproduction in *Aspergillus flavus* Sclerotia: Acquisition of Novel Alleles from Soil Populations and Uniparental Mitochondrial Inheritance

Published by PLoS One

Bruce W. Horn[1], Richard M. Gell[2], Rakhi Singh[2], Ronald B. Sorensen[1], Ignazio Carbone[2]*

[1]National Peanut Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Dawson, Georgia, United States of America. [2]Center for Integrated Fungal Research, Program of Genetics, Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina, United States of America.

*E-mail: icarbon@ncsu.edu

CONTRIBUTION SUMMARY

For the experiments within this paper, I was involved with the planning, execution, and analysis of the genotyping steps. I designed the sampling approach for the strains with Ignazio Carbone including primer choice and design where necessary. I conducted the DNA extractions, PCR amplifications and gel electrophoresis with Rakhi Singh. I assisted with writing and editing the areas relevant to where I conducted the experimental portion.

ABSTRACT

Aspergillus flavus colonizes agricultural commodities worldwide and contaminates them with carcinogenic aflatoxins. The high genetic diversity of *A. flavus* populations is largely due to sexual reproduction characterized by the formation of ascospore-bearing ascocarps embedded within sclerotia. *A. flavus* is heterothallic and laboratory crosses between strains of the opposite mating type produce progeny showing genetic recombination. Sclerotia formed in crops are dispersed onto the soil surface at harvest and are predominantly produced by single strains of one mating type. Less commonly, sclerotia may be fertilized during co-infection of crops with sexually compatible strains. In this study, laboratory and field experiments were performed to examine sexual reproduction in single-strain and fertilized sclerotia following exposure of sclerotia to natural fungal populations in soil. Female and male roles and mitochondrial inheritance in *A. flavus* were also examined through reciprocal crosses between sclerotia and conidia. Single-strain sclerotia produced ascospores on soil and progeny showed biparental inheritance that included novel alleles originating from fertilization by native soil strains. Sclerotia fertilized in the laboratory and applied to soil before ascocarp formation also produced ascospores with evidence of recombination in progeny, but only known parental alleles were detected. In reciprocal crosses, sclerotia and conidia from both strains functioned as female and male, respectively, indicating *A. flavus* is hermaphroditic, although the degree of fertility depended upon the parental sources of sclerotia and conidia. All progeny showed maternal inheritance of mitochondria from the sclerotia. Compared to *A. flavus* populations in crops, soil populations would provide a higher likelihood of exposure of sclerotia to sexually compatible strains and a more diverse source of genetic material for outcrossing.

INTRODUCTION

Aflatoxins produced by *Aspergillus flavus* from section *Flavi* are among the most potent mycotoxins known. These secondary metabolites are acutely toxic to humans at high exposures and are also responsible for increased incidences of liver cancer in human populations in which contaminated food is routinely ingested [1,2]. Aflatoxin-producing fungi were originally thought to be strictly asexual in reproduction and to have lost their ability to undergo meiosis [3]. However, populations of *A. flavus* show high diversity in morphology, mycotoxin production and vegetative compatibility groups (VCGs) [4,5]. In addition, *A. flavus* populations exhibit evolutionary signatures of recombination within the aflatoxin gene cluster based on the partitioning of DNA sequence variation into distinct linkage disequilibrium blocks [6,7]. The discovery of sexual reproduction in *A. flavus* [8] in laboratory crosses as well as the demonstration of independent assortment of chromosomes and crossing over [9,10] suggest that sexuality is largely responsible for the genetic variation observed in natural populations. For example, many *A. flavus* strains in populations do not produce aflatoxins due to specific deletions in the aflatoxin gene cluster [11]. The locations of these deletions were shown to correspond to cross over points during meiosis in laboratory crosses [9]. Therefore, sexual reproduction and genetic recombination in nature may be responsible for the genetic variation among nonaflatoxigenic *A. flavus* strains.

A. flavus is heterothallic, with individuals containing one of two mating-type alleles, *MAT1-1* and *MAT1-2* [7,12]. Sexual reproduction in crosses between opposite mating types is characterized by the formation of indehiscent ascospore-bearing ascocarps within the matrix of sclerotia [8]. In many fungi, sexual reproduction is also regulated by a sex-based (female/male) mating system independent of mating type [13-15], but such as system has not been reported in

A. flavus. Two morphotypes of *A. flavus* based on sclerotial size have been described: the L (large) strain with sclerotia > 400 µm diam and the S (small) strain with sclerotia < 400 µm [16]. Sclerotia are readily produced by single strains in culture [5] and in wound-inoculated crops [17,18], and their formation is not dependent on mating; hence, they are primarily considered to be survival structures for withstanding adverse environmental conditions [19].

Sclerotia of *A. flavus* are naturally produced in crops [20,21] and are dispersed onto the soil surface during harvest [20]. The majority of these sclerotia likely originate from single strains of one mating type. To examine the capacity of naturally formed *A. flavus* sclerotia to produce the sexual stage, Horn et al. [21] collected sclerotia from corn exposed to different levels of drought stress over a 3-year period. There was no evidence of ascocarp and ascospore formation in sclerotia at corn harvest, but incubation of sclerotia on the surface of soil in the laboratory resulted in ascospore formation in a very small percentage of sclerotia. Horn et al. [21] postulated that fertilization occurred in the crop and that the development of sexual structures occurred after dispersal of sclerotia onto the soil surface at harvest. The low incidence of sexual reproduction in *A. flavus* sclerotia was attributed to the low probability of co-infection of corn with sexually compatible strains. Therefore, although fertilized sclerotia may be dispersed onto soil, the majority of sclerotia will be unfertilized and consist of single strains.

In this study, laboratory and field experiments were performed to examine the capacity of single-strain sclerotia of one mating type and fertilized sclerotia that had not yet formed ascocarps to produce ascospores on soil containing natural fungal populations. Progeny were examined for recombination and the presence of novel parental alleles. Reciprocal crosses between sclerotia and conidia were also performed to investigate female and male roles and mitochondrial inheritance in *A. flavus* sexual reproduction. This research shows that both single-

strain and fertilized sclerotia can undergo sexual development on soil, but progeny from single-strain sclerotia contain novel alleles from fertilization by soil strains, whereas progeny from fertilized sclerotia contain only known parental alleles. Furthermore, reciprocal crosses between sclerotia and conidia show that *A. flavus* is hermaphroditic with respect to female and male roles in sexual reproduction and that inheritance of mitochondria is uniparental.

MATERIALS and METHODS

Fungal strains and sclerotium production

A. flavus L strains used to produce sclerotia (Table 1) were chosen based on mating type and high fertility in laboratory crosses [8,9,12]. Nonaflatoxigenic biocontrol strains NRRL 21882 from Afla-Guard and AF36 (= NRRL 18543), both used commercially for reducing aflatoxins in crops [22], were assigned to VCGs according to Horn and Dorner [23] and Ehrlich et al. [24], respectively. The remaining strains were obtained from soil and peanut seeds from a field (private land with permission) in Terrell Co., Georgia (31°41'39"N 84°25'00"W), and were previously characterized by VCG [4]; all strains produce aflatoxin B_1 and cyclopiazonic acid [5].

For producing sclerotia, slants containing mixed cereal agar (MCA) [25] were inoculated with conidia from either single strains or pairs of strains in crosses according to Horn et al. [26]. Cultures were incubated in darkness for 14 d at 30 °C, at which time fertilized sclerotia from crosses had not yet formed ascocarps. Since the fertilization process has not been observed in *A. flavus*, sclerotia were considered to be fertilized based on the subsequent development of ascocarps with ascospores under laboratory conditions. In all crosses, a certain percentage of sclerotia did not form the sexual stage. Sclerotia from single strains and crosses were harvested and then air dried and stored in a desiccator over saturated NaCl solution at 25 °C (75% relative humidity) [26]. Following a subsequent incubation period in the laboratory or field, sclerotia were surface sterilized, dissected with a microscalpel, and examined for ascocarps with the stereomicroscope. To obtain progeny, ascospores were removed from individual ascocarps with a microneedle and dilution plated on malt extract agar containing 30 mg/L streptomycin and 1.5 mg/L chlortetracycline [26]. Germlings were observed with the light microscope (200×) after 20-24 h incubation at 30 °C and transferred to Czapek agar (CZ).

Laboratory incubation of single-strain sclerotia on soil

The capacity of single-strain sclerotia of one mating type to produce ascospores through incubation on soil containing natural soil populations was examined under laboratory conditions. Soil was collected 17 March 2011 from a cornfield 1.1 km southeast of Shellman, Randolph Co., Georgia (Field A) (31°44’47”N 84°36’22”W). Soil sampling and field trials were conducted on property owned or leased by ARS-USDA. Soil was air dried to 1.6 ± 0.01% moisture (± SD, n = 3; dry weight basis) and sieved through No. 12 and 20 Standard Testing Sieves in tandem. Potential mating population densities of *A. flavus* in soil were determined by suspending each of three subsamples (33 g) in 100 mL 0.2% water agar and dilution plating onto five plates of modified dichloran-rose Bengal medium (mDRB) [27]. Plates were incubated for 3 d at 37 °C and section *Flavi* species were identified according to Horn and Dorner [27]. Soil population densities were calculated on a dry-weight soil basis. In addition, 37 randomly selected *A. flavus* L strain colonies from soil dilution plates were single spored for mating-type determination.

Sieved soil was mixed with sterile distilled water (14 mL per 100 g) and allowed to equilibrate overnight in a sealed container before adding to 30-cm^3 plastic medicine cups [21]. *A. flavus* sclerotia from each of seven single strains (Table 1) were added to the soil surface of three medicine cups (approximately 300 per cup). Sclerotia were similarly added to cups containing autoclaved soil to which sterile water (14 mL per 100 g) had been added. Cups were incubated separately in darkness for 6 mo on shallow platforms over distilled water in wide-mouth sealed quart jars (30 °C; 100% relative humidity).

Field incubation of single-strain and fertilized sclerotia

The capacity to produce ascospores by single-strain sclerotia of one mating type and fertilized sclerotia that had not yet formed ascocarps was examined in three fields. Single-strain and fertilized sclerotia were applied to the soil surface of three non-irrigated cultivated fields in Georgia: Field A, described above near Shellman; Field B, 4.8 km northwest of Dawson, Terrell Co. (31°47’06”N 84°29’16”W); and Field C, 4.8 km southeast of Dawson, Terrell Co. (31°43’59”N 84°23’37”W). Soil from Field B was Faceville fine sandy loam (fine, kaolinitic, thermic Typic Kandiudults) and soils from Fields A and C were Greenville fine sandy loam (fine, kaolinitic, thermic Rhodic Kandiudults). Analyses for soil texture, organic matter and pH were performed by Waters Agricultural Laboratories, Camilla, GA. Rainfall and air temperatures were recorded from onsite electronic weather stations (Campbell Scientific, Logan, UT). Daily minimum, maximum and mean temperature values for each month (n = 28-31 except n = 15 for April) were statistically compared among fields with ANOVAs followed by Student-Newman-Keuls (SNK) test for comparison of means.

A 16 × 6 m plot was delimited and fenced within each field. Within each plot, 13 circular white PVC rings (15.2 cm diam and deep) were spaced approximately 2 m apart and inserted 7-8 cm into the soil. To determine potential mating population densities of *A. flavus* in the plot soils, five soil samples (33 g each) from the top 3 cm were randomly collected outside of the rings from each plot on 16 Apr 2013 (immediately before applying sclerotia) and were dilution plated on mDRB as described above. Population densities of section *Flavi* species in the three fields were statistically compared with ANOVAs followed by SNK test for comparison of means. Twelve randomly selected *A. flavus* L strain colonies from each field were single spored for mating-type determination.

Single-strain sclerotia and fertilized sclerotia from crosses were harvested from MCA slants (14 d; 30 °C) for applying to the fields. Previous research [21] demonstrated that *A. flavus* sclerotia from crosses did not contain ascocarps after 14 d. However, a certain percentage of sclerotia contained ascocarps with free ascospores after 4 mo when sclerotia were incubated in MCA slants and on soil in the laboratory. To ensure that sclerotia from crosses in this study were also capable of forming the sexual stage when applied to the fields, sclerotia in MCA slants were incubated an additional 4 mo at 30 °C in sealed plastic bags [26] and sclerotia harvested from MCA slants (14 d; 30 °C) were incubated on the surface of nonsterile soil in cups within fruit jars for 4 mo at 30 °C as described above. Approximately 1000-1500 sclerotia from each of seven single strains and six sexually compatible crosses (Table 2) were sprinkled onto the soil surface within randomly selected rings. Distilled water (100 mL) was applied to each ring with a watering can immediately following application of sclerotia; thereafter, sclerotia were exposed wholly to rainfall. Pesticides were not applied to the plots, and weeds were allowed to grow freely to form a canopy over the rings.

The top 2 cm of soil was removed from the entire area within each ring after incubation of sclerotia for one year (April 2014). Soil was added to a 1-L graduated cylinder and brought to a final volume of 1 L with 2.5 M sucrose [28]. After vigorously shaking the cylinder, sclerotia and other organic matter were allowed to float to the top of the sucrose solution (3 h). The floating fraction was then transferred to a 100-mesh filter, rinsed with distilled water, and retransferred to 9-cm Whatman #1 filter paper from which sclerotia were removed using a stereomicroscope. Sclerotia were surface sterilized for 2 min with 0.25% sodium hypochlorite and rinsed before dissection. Dissected sclerotia without ascocarps from each ring (n = 49-75) were plated on CZ with antibiotics to test for viability.

Reciprocal crosses between single-strain sclerotia and conidia

Reciprocal crosses between sclerotia and conidia were performed in the laboratory to examine female and male roles in sexual reproduction and to determine the pattern of mitochondrial inheritance. *A. flavus* sclerotia from single strains of one mating type were added to medicine cups containing autoclaved sieved soil to which conidia of the opposite or same mating type had been added. Conidia were obtained by inoculating slants containing CZ with 400g/L sucrose and incubating in darkness for 14 d at 30 °C. Sterile glass beads (2.5 g; 90-150 µm diam) were then added to each slant and shaken to coat the beads with conidia [29]. To effectively mix the conidia in soil, coated beads (0.09 g) for each strain were added to a jar containing 600 g of dry autoclaved soil and thoroughly shaken. Inoculated soil (3.3 g) was added to 10 mL water agar and dilution plated on mDRB plates (3 d; 37 °C) to determine fungal density; soil in jars was then adjusted with sterile soil or conidia-coated beads to attain approximately 2000 CFU/g. Sterile water was added to the inoculated soil (14 mL per 100 g) and sclerotia were incubated in soil cups within sealed fruit jars for 6 mo at 30 °C as described above. Three pairs of reciprocal sclerotia-conidia combinations (Table 3) were set up using crosses that produced sclerotia with high fertility when incubated 4 mo in MCA slants and soil cups (Table 4). For each reciprocal pair, sclerotia of the *MAT1-1* strain were incubated on soil containing conidia of the *MAT1-2* strain, and sclerotia of the *MAT1-2* strain were incubated on soil containing conidia of the *MAT1-1* strain. In addition, sclerotia of the two strains from each reciprocal pair were incubated with conidia of a strain of the same mating type (*MAT1-1* or *MAT1-2*).

Mating-type determination and genotype analyses

DNA was extracted from *A. flavus* soil and progeny strains as previously described [30]; previously generated sequence data [9] were used for parental strains. Mating types for strains were determined according to Ramirez-Prado et al. [12]. Deletion types for the *aflF-aflU* region were determined according to Chang et al. [11]. PCR amplification for the *AF17* and *AF48* microsatellite loci was performed based on Grubisha and Cotty [31] and for *aflC* based on Moore et al. [6]. Oligonucleotides for *AF-MIT-1* (F: TGAAGCAACTGGATTATTCGCA, R: AAACCACATTCAAAAGCGCT), *AF-MIT-3* (F: AGCAGAGGGTTCTGCGTTT, R: GCAGATCAACCTGCTAATAATATTCC) and *AF-MIT-4* (F: GCTAAAGTTATAGGAGGTGAAGT, R: GCAACCTTTAGCTTCAATAAACCC) were designed for amplification of polymorphic loci in the mitochondrial genomes of parental and progeny strains. Nuclear *AF17, AF48* and *aflC* and mitochondrial amplicons were sequenced by the North Carolina State University Genomic Sciences Laboratory and aligned using SEQUENCHER version 4.7 (Gene Codes Corporation, Ann Arbor, MI). Haplotypes were designated based on single-nucleotide polymorphisms, insertion/deletion events and trinucleotide repeat lengths using the SNAP Map and Combine programs [32] implemented in Mobyle SNAP Workbench [33,34].

Three ascocarps from different sclerotia, when possible, were randomly chosen from single-strain sclerotia that became fertilized following incubation on soil in the laboratory (NRRL 29507, NRRL 29473, NRRL 29536) or in the field (NRRL 29507, NRRL 29473). Three progeny strains from each ascocarp were analyzed at the *MAT* locus on chromosome 6, *AF17* locus on chromosome 2, *AF48* locus on chromosome 7, and *aflF-aflU* intergenic region on chromosome 3. For fertilized sclerotia obtained from crosses between known parental strains

(NRRL 29507 × AF36, NRRL 29473 × AF36, NRRL 29507 × NRRL 21882) and incubated under field conditions, two ascocarps from different sclerotia were randomly chosen per field. Three progeny strains from each ascocarp were analyzed at the *MAT*, *AF17*, *AF48* and *aflC* (chromosome 3) loci. For the three pairs of reciprocal crosses between sclerotia and conidia from known parents, three ascocarps per cross from different sclerotia were chosen. Two progeny strains per ascocarp were analyzed at the *MAT* locus and a mitochondrial marker (*AF-MIT-1* for NRRL 29537 and NRRL 29536; *AF-MIT-3* for NRRL 29507 and NRRL 21882; and *AF-MIT-4* for NRRL 29473 and AF36).

RESULTS

Laboratory incubation of single-strain sclerotia on soil

Soil used for laboratory incubation of sclerotia from single strains of one mating type contained a sizable potential mating population of *A. flavus* L strain. Section *Flavi* species included: *A. flavus* L strain (777 ± 216 CFU/g; ± SD, n = 3), *A. flavus* S strain (11 ± 4), *A. parasiticus* (149 ± 27), *A. caelatus* (508 ± 117) and *A. tamarii* (14 ± 6). Of the 37 *A. flavus* L strains randomly sampled from the soil dilution plates, 19 (51%) were *MAT1-1* and 18 (49%) were *MAT1-2*.

Sclerotia from three *MAT1-1* and four *MAT1-2* single strains of *A. flavus* were incubated for 6 mo on the surface of sterile soil and soil containing natural fungal populations (Table 1). Sclerotia incubated on sterile soil showed no evidence of ascocarp formation, whereas sclerotia from five of the strains (NRRL 29507, NRRL 29473, NRRL 29537, NRRL 29536 and NRRL 21882) incubated on soil with natural fungal populations showed ascospore formation in 0.1-80.2% of sclerotia; two of the strains (NRRL 29487 and AF36) did not produce ascocarps (Table 1, S1 Text). Of the progeny examined from single-strain sclerotia (NRRL 29507, NRRL 29473, NRRL 29536), only 2 of 27 (IC5210 and IC5958) showed multilocus sequence types (MLSTs) that matched the known sclerotial parent (Table 5). The remaining progeny showed biparental inheritance with independent assortment of chromosomes and contained novel alleles from wild strains in soil. Sequence polymorphisms in *AF17* and *AF48* identified the novel alleles contributed by wild parents. Progeny from each of the nine ascocarps examined showed inheritance from a single wild strain; ascocarps 1 and 3 from NRRL 29536 had MLSTs consistent with the same wild parental strain (Table 5). Both mating-type alleles (*MAT1-1/MAT1-2*) were detected in three of the progeny. Progeny strains from the incubation of NRRL

21882 sclerotia could not be conclusively genotyped due to the presence of multiple alleles inherited from the known parental strain and wild strains. All sequence data for *AF17* and *AF48* were submitted to GenBank under Accession numbers KR922515- KR922572.

Field incubation of single-strain and fertilized sclerotia

Soil within each field plot immediately prior to application of single-strain and fertilized sclerotia contained native populations of *A. flavus* L strain potentially capable of fertilizing the sclerotia. Populations from section *Flavi* included *A. flavus* L and S strains, *A. parasiticus*, *A. tamarii*, *A. caelatus* and *A. alliaceus* (Table 6). *A. flavus* L strain densities in Fields A and C (398 and 391 CFU/g, respectively) were not significantly different (P = 0.96), whereas the density in Field B (8135 CFU/g) was significantly greater than those of Fields A and C (P < 0.0001). Mating types of randomly selected isolates (n = 12) from soil were: 7 (58%) *MAT1-1* and 5 (42%) *MAT1-2* for Field A; 6 (50%) *MAT1-1* and 6 (50%) *MAT1-2* for Field B; and 2 (17%) *MAT1-1*, 9 (75%) *MAT1-2* and 1 (8%) *MAT1-1/MAT1-2* for Field C. Sclerotia within rings in the plots were initially unshaded when applied in April 2013 but were covered by weeds by June.

Soil analyses for Field B showed 81.2% sand, 10.4% clay, 8.4% silt and 0.8% organic matter; Fields A and C were similar for percentage sand (67.2 and 69.2, respectively), clay (20.0, 20.4), silt (12.8, 10.4) and organic matter (1.2, 1.0). The pH of soil from the three fields was 6.6-7.2. During the course of the experiment, monthly maximum, minimum and mean air temperatures (S1 Table) showed no significant differences (P > 0.05) among the three fields, with the exception of minimum temperature in July 2013 in which Field A was significantly lower (P < 0.0001) than Fields B and C. Sclerotia in the three fields were exposed to air temperatures below freezing for 3-5 d in Nov, 4 d in Dec, 18-19 d in Jan, 5-6 d in Feb and 1-2 d

in March. Rainfall totals for one year following application of sclerotia to Fields A, B and C were 151.4, 146.0 and 167.0 cm, respectively (S1 Table). Monthly variations in rainfall among fields were primarily due to thunderstorms in which rainfall can be intense and localized.

Single-strain sclerotia of one mating type when dissected after one year in the field showed a low frequency of ascospore formation (≤ 1.2%) in Field B but not Fields A and C (Table 2, S1 Text). Ascospores were present in sclerotia of three *MAT1-1* strains (NRRL 29507, NRRL 29473, NRRL 29537). Viability of sclerotia that did not form ascocarps in the three fields ranged from 38.7 to 98.7%; nonviable sclerotia were often colonized by *Fusarium* species. Sclerotia of NRRL 29507 and NRRL 29473 produced relatively few progeny strains that matched the MLST of the known parental strain; three of the progeny contained both mating-type alleles (*MAT1-1/MAT1-2*) (Table 7). In the majority of progeny, MLSTs indicated biparental inheritance and independent assortment of chromosomes. Sequence polymorphisms in *AF17* and *AF48* distinguished novel alleles contributed by wild parents from soil. Each ascocarp showed inheritance from a single wild strain; progeny from ascocarps 2A and 2B within the same sclerotium of NRRL 29473 appear to have originated from fertilization by the same wild strain (Table 7).

Fertilized sclerotia from all six crosses did not contain ascocarps when harvested from MCA slants (14 d; 30 C) prior to application to the three fields (Table 4). In all crosses, a certain percentage of sclerotia were capable of sexual reproduction at the time of field application. Sclerotia formed ascospores after extended incubation (4 mo) in MCA slants (14.7-91.5% fertility) and on the surface of nonsterile soil under laboratory conditions (24.9-78.3%) (Table 4). Fertilized sclerotia from laboratory crosses, with the exceptions of NRRL 29537 × 29536 (Fields A-C) and NRRL 29473 × 21882 (Field A), showed low frequencies of ascospore

formation in the three fields (Table 2). In all fields, sclerotia from NRRL 29507 × 21882 were most fertile (6.2-22.7%) followed by NRRL 29473 × AF36 (3.3-9.0%) and NRRL 29507 × AF36 (1.2-2.9%). When incubated in MCA slants and soil cups, sclerotia from these three crosses also showed significantly higher fertilities than sclerotia from the other crosses when compared with the chi-square test of independence ($P < 0.01$), except for NRRL 29507 × 21882 compared to NRRL 29537 × NRRL 29536 when incubated in MCA slants ($P = 0.17$) (Table 4, S1 Text). Viability of sclerotia that did not form ascocarps in the three fields ranged from 18.7 to 96.0% (Table 2). Fertile ascocarps from sclerotia of NRRL 29507 × AF36, NRRL 29473 × AF36 and NRRL 29507 × NRRL 21882 in all three fields produced progeny showing independent assortment of chromosomes (Table 8). Progeny strains from the laboratory-fertilized sclerotia contained only known parental alleles; no novel alleles from wild strains were detected (Table 8). Sequence data for *aflC*, *AF17* and *AF48* were submitted to GenBank under Accession numbers KR922585- KR922776.

Reciprocal crosses and mitochondrial inheritance

Reciprocal crosses between sclerotia and conidia were performed to elucidate female and male roles in sexual reproduction and to determine the pattern of mitochondrial inheritance. *A. flavus* sclerotia from single strains of one mating type formed ascospore-bearing ascocarps when incubated on the surface of sterilized soil inoculated with conidia of the opposite mating type, indicating that sclerotia functioned as female and conidia served as a male (Table 3). Hermaphroditism was shown by the formation of ascospores in sclerotia of both strains within each reciprocal cross. In the crosses NRRL 29507 × 21882 and NRRL 29473 × AF36, sclerotia from NRRL 29507 and NRRL 29473 (*MAT1-1*) readily formed ascospores when incubated with respective NRRL 21882 and AF36 (*MAT1-2*) conidia in the soil. However, fertility was

significantly lower in reciprocal combinations in which NRRL 21882 and AF36 (*MAT1-2*) sclerotia were incubated with respective NRRL 29507 and NRRL 29473 (*MAT1-1*) conidia when compared by chi-square test for independence ($P < 0.0001$) (Table 3, S1 Text). In contrast, NRRL 29537 × 29536 showed markedly higher fertility when NRRL 29536 (*MAT1-2*) sclerotia were incubated with NRRL 29537 (*MAT1-1*) conidia compared to the reciprocal combination ($P < 0.0001$). None of the sclerotia-conidia combinations involving *MAT1-1* × *MAT1-1* and *MAT1-2* × *MAT1-2* produced ascocarps (Table 3).

The progeny from sclerotia-conidia crosses NRRL 29537 × NRRL 29536, NRRL 29507 × NRRL 21882 and NRRL 29473 × AF36 and their reciprocal combinations inherited the mitochondrial genome, as indicated by markers *AF-MIT-1*, *AF-MIT-3* and *AF-MIT-4*, respectively, from the sclerotial parent (Table 9). All sclerotia-conidia crosses exhibited segregation in the nuclear genome (*MAT* locus) of progeny (Table 9). Both mating-type alleles (*MAT1-1/MAT1-2*) were detected in eight of the progeny. Sequence data for mitochondrial *AF-MIT-1*, *AF-MIT-3* and *AF-MIT-4* were submitted to DRYAD database with accession DOI: http://dx.doi.org/10.5061/dryad.sk35h.

DISCUSSION

This research suggests that *A. flavus* is versatile in the manner in which sclerotia are fertilized and reproduce sexually on the soil surface. Single-strain sclerotia of one mating type, which appear to predominate in nature, can be fertilized by strains in native soil populations after dispersal from the crop. Furthermore, fertilized sclerotia without ascocarps, which originate from crops co-infected with sexually compatible strains, can also form the sexual stage after dispersal. Sexual reproduction in both single-strain and fertilized sclerotia was observed under laboratory conditions when incubated on soil containing natural fungal populations (Tables 1 and 4) and under field conditions on soil after one year (Table 2). Progeny from single-strain sclerotia exposed to natural soil populations in the laboratory and field showed the acquisition of novel alleles from outcrossing with soil strains as well as recombination (independent assortment of chromosomes) (Tables 5 and 7). In contrast, progeny from fertilized sclerotia applied to fields before ascocarp formation showed only known parental alleles (Table 8). Prior fertilization of sclerotia may have prevented additional fertilization events, as illustrated in basidiomycetes following dikaryon formation [35].

Outside of the present study, there are no known examples among Aspergilli in which a mature sclerotium is capable of being fertilized by natural soil populations. Additional research is needed to determine the nature of the soil propagule (conidium or hypha) and whether direct contact of the sclerotium with the propagule is required or chemotrophic growth of the propagule through soil to the sclerotium is involved. Receptor structures for fertilization have not been detected on the surface of *A. flavus* sclerotia. Heterothallic *Botrytis cinerea* commonly produces sclerotia that function as survival structures [36]. These sclerotia can also serve as a female parent but unlike *A. flavus*, *B. cinerea* produces specialized microconidia (spermatia) for

fertilization [37]. Heterothallic *Epichloë* species produce conidia that act as spermatia for fertilizing immature stromata on grasses, but the conidia are transmitted primarily by insects and originate from other stromata rather than soil populations [38].

Laboratory incubation of single-strain sclerotia on soil resulted in wide variation in fertility despite an equal proportion of mating types in soil, with some strains readily producing ascospores and others showing little or no evidence of sexual reproduction (Table 1). This variation might be attributed to female fertility factors in the strains producing the sclerotia (see below), but also could be influenced by the degree of sexual compatibility between sclerotia and soil strains independent of mating type. Sexual reproduction in *Aspergillus* in general is regulated by over 70 genes at different stages of development [39]. Sexual incompatibility is typically due to an accumulation of mutations of these genes [40,41] and is expected to be most prevalent in asexual fungi that undergo extensive genetic drift [14,42,43]. *A. flavus* is predominantly asexual and populations comprise numerous clonal lineages of varying degrees of genetic relatedness [6,7,44]. Genetic incompatibilities among lineages in *A. flavus* may be partially responsible for the *MAT1-1* × *MAT1-2* crosses that exhibit low fertility or do not produce viable progeny [8,9]. In this study, the biocontrol strains NRRL 21882 and AF36 showed extremely low fertility when sclerotia were incubated on soil under laboratory and field conditions (Tables 1 and 2, respectively) but showed relatively high fertility when sclerotia were fertilized in specific crosses in culture slants (Table 4). Therefore, the low fertility with soil incubations may have been due to the genetic composition of the *A. flavus* populations, and exposure to different soil populations might result in higher fertility. A number of *A. flavus* progeny from laboratory and field experiments showed both *MAT1-1* and *MAT1-2* (Tables 5, 7 and 9). One such *MAT1-1/MAT1-2* progeny strain from a laboratory cross also was reported by

Olarte et al. [9]. The mechanism responsible for the presence of both mating types is not understood and additional research is required to determine whether heterokaryosis, B chromosomes or ectopic plasmids are involved.

In both single-strain and fertilized sclerotia, incubation on soil with natural fungal populations showed much higher frequencies of ascospore formation under laboratory conditions of high relative humidity (100%) and a constant temperature (30 °C) compared to suboptimal conditions in the fields. All three fields were exposed to similar temperatures (S1 Table) and contained *A. flavus* soil populations with both *MAT1-1* and *MAT1-2* strains. Furthermore, the three fields showed similar frequencies of ascospore formation in fertilized sclerotia from crosses (Table 2), suggesting that environmental conditions were similar and that differences in soil type (Faceville fine sandy loam in Field B and Greenville fine sandy loam in Fields A and C) had little effect. Despite the many similarities in field conditions, ascospore formation in single-strain sclerotia was observed at low frequencies only from Field B (Table 2). The most prominent difference among fields involved *A. flavus* L strain population densities in soil, with the density in Field B (8135 CFU/g) being approximately 20× higher than the densities in Fields A and C (Table 6). A high population density could increase the likelihood of sclerotium fertilization and account for the detection of sexual reproduction only in Field B.

Little is known about the mechanism of fertilization and the subsequent development of ascocarps, asci and ascospores in sclerotia of *A. flavus* and other section *Flavi* species. Gametangia have been reported in several *Aspergillus* species [45] but not in section *Flavi*. Homothallic *A. alliaceus* from section *Flavi* produces sclerotia whose matrix consists of thick-walled pseudoparenchymatous cells [46-48]. Fennell and Warcup [46] reported ‘channeling’ within the matrix immediately prior to the appearance of ascocarps. Transmission electron

microscopy revealed that the channeling may be due to the interspersion of groups of cells containing cytoplasm with other groups of cells in various stages of autolysis [49]. Intracellular hyphae were also observed and may be involved in the early stages of ascocarp formation. In *A. flavus*, the matrix of mature sclerotia also consists of thick-walled pseudoparenchymatous cells with cytoplasmic contents [50]. Wada et al. [51] reported on the formation of heterokaryotic sclerotia in *A. oryzae*, a species often considered to be con-specific with *A. flavus* [52]. However, in that study, nutritional mutants were paired and sclerotia were produced independent of mating-type combination and only by strains with different auxotrophies.

In reciprocal crosses, sclerotia and conidia from both strains within each cross functioned as female and male, respectively, indicating *A. flavus* is hermaphroditic. The sclerotium, as a source of nutrients for sexual development, can be considered functionally female and the conidia used for fertilization functionally male. The degree of fertility depended upon the parental source of sclerotia and conidia (Table 3). In each reciprocal cross, one sclerotia-conidia combination was highly fertile while the reciprocal combination produced a significantly lower frequency ($P < 0.0001$) of sclerotia containing ascospore-bearing ascocarps. These results concur with the presence of fungal populations with varying proportions of strains that are hermaphroditic or female sterile [13,14,53], with three of the strains in this study (NRRL 29537, NRRL 21882, AF36) approaching female sterility (Table 3). The combinations leading to highest fertility involved sclerotia from both *MAT1-1* and *MAT1-2* strains, indicating that the gender-based sexual system is independent of the mating-type compatibility system [13]. Strain-dependent differential expression of sex-based genes necessary for fertilization and sexual development by female and male parents [54] could account for these differences and further work should be done to identify any causal link. Only three reciprocal sclerotia-conidia crosses

of *A. flavus* were examined in this study and additional reciprocal crosses might reveal pairs of strains that show equal female fertility or strains in which one or both are completely female sterile.

Among the three pairs of reciprocal crosses between sclerotia and conidia, the sclerotial parent contributed the mitochondrial genome to progeny (Table 9). Such uniparental inheritance of mitochondria from the female parent is the most prevalent pattern in fungi [55]. Many anisogamous ascomycetes are characterized by an ascospore-bearing female whose nuclei are contributed to the ascocarp wall and whose nuclei and mitochondria are contributed to ascospores, and by a male whose nuclei are contributed solely to ascospores [14]. Outcrossing between strains in homothallic *A. nidulans* reveals such female and male roles in nuclear and mitochondrial inheritance during sexual reproduction [15] and is consistent with observations in *A. flavus*.

In conclusion, the current study helps to elucidate the mechanisms available to *A. flavus* for sexual reproduction in natural environments. Both proposed mechanisms—fertilization of single-strain sclerotia by native soil populations and fertilization of sclerotia in crops before dispersal onto soil—are supported by this research, though questions remain concerning their relative importance in nature. In addition, female fertility in *A. flavus*, as indicated through the reciprocal crosses, requires additional research to determine its role in regulating sexual reproduction and how it interacts with mating type in influencing population structure. Soil populations of *A. flavus* are highly diverse genetically [4,5,7]. Sweany et al. [56] reported that *A. flavus* populations in corn from 11 Louisiana fields comprised relatively few VCGs and haplotypes and were mostly *MAT1*-2, whereas soil populations from those same cornfields comprised a large number of VCGs and haplotypes and an equal proportion of mating types.

Therefore, compared to *A. flavus* populations in crops, soil populations would provide a higher likelihood of exposure of sclerotia to sexually compatible strains and a more diverse source of genetic material for outcrossing.

REFERENCES

1. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D (2004) Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80: 1106-1122.

2. Kew MC (2013) Aflatoxins as a cause of hepatocellular carcinoma. J Gastrointestin Liver Dis 22: 305-310.

3. Geiser DM, Timberlake WE, Arnold ML (1996) Loss of meiosis in *Aspergillus*. Mol Biol Evol 13: 809-817.

4. Horn BW, Greene RL (1995). Vegetative compatibility within populations of *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii* from a peanut field. Mycologia 87: 324-332.

5. Horn BW, Greene RL, Sobolev VS, Dorner JW, Powell JH, Layton RC (1996) Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii*. Mycologia 88: 574-587.

6. Moore GG, Singh R, Horn BW, Carbone I (2009). Recombination and lineage-specific gene loss in the aflatoxin gene cluster of *Aspergillus flavus*. Mol Ecol 18: 4870-4887.

7. Moore GG, Elliott JL, Singh R, Horn BW, Dorner JW, Stone EA, et al. (2013) Sexuality generates diversity in the aflatoxin gene cluster: evidence on a global scale. PLoS Pathog 9: e1003574.

8. Horn BW, Moore GG, Carbone I (2009) Sexual reproduction in *Aspergillus flavus*. Mycologia 101: 423-429.

9. Olarte RA, Horn BW, Dorner JW, Monacell JT, Singh R, Stone EA, et al. (2012) Effect of sexual reproduction on population diversity in aflatoxin production by *Aspergillus flavus* and evidence for cryptic heterokaryosis. Mol Ecol 21: 1453-1476.

10. Olarte RA, Worthington CJ, Horn BW, Moore GG, Singh R, Monacell JT, et al. (2015) Enhanced diversity and aflatoxigenicity in interspecific hybrids of *Aspergillus flavus* and *Aspergillus parasiticus*. Mol Ecol 24: 1889-1909.

11. Chang P-K, Horn BW, Dorner JW (2005) Sequence breakpoints in the aflatoxin biosynthetic gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. Fungal Genet Biol 42: 914-923.

12. Ramirez-Prado JH, Moore GG, Horn BW, Carbone I (2008) Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. Fungal Genet Biol 45: 1292-1299.

13. Leslie JF (1995) *Gibberella fujikuroi*: available populations and variable traits. Can J Bot 73: S282-S291.

14. Leslie JF, Klein KK (1996) Female fertility and mating type effects on effective population size and evolution in filamentous fungi. Genetics 144: 557-567.

15. Bruggeman J, Debets AJM, Swart K, Hoekstra RF (2003) Male and female roles in crosses of *Aspergillus nidulans* as revealed by vegetatively incompatible parents. Fungal Genet Biol 39: 136-141.

16. Cotty PJ (1989) Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 78: 808-814.

17. Wicklow DT, Horn BW (1984) *Aspergillus flavus* sclerotia form in wound-inoculated preharvest corn. Mycologia 76: 503-505.

18. Garber RK, Cotty PJ (1997) Formation of sclerotia and aflatoxins in developing cotton bolls infected by the S strain of *Aspergillus flavus* and potential for biocontrol with an atoxigenic strain. Phytopathology 87: 940-945.

19. Wicklow DT (1987) Survival of *Aspergillus flavus* sclerotia in soil. Trans Br Mycol Soc 89: 131-134.

20. Wicklow DT, Horn BW, Burg WR, Cole RJ (1984) Sclerotium dispersal of *Aspergillus flavus* and *Eupenicillium ochrosalmoneum* from maize during harvest. Trans Br Mycol Soc 83: 299-303.

21. Horn BW, Sorensen RB, Lamb MC, Sobolev VS, Olarte RA, Worthington CJ, et al. (2014) Sexual reproduction in *Aspergillus flavus* sclerotia naturally produced in corn. Phytopathology 104: 75-85.

22. Abbas HK, Zablotowicz RM, Horn BW, Phillips NA, Johnson BJ, Jin X, et al. (2011) Comparison of major biocontrol strains of non-aflatoxigenic *Aspergillus flavus* for the reduction of aflatoxins and cyclopiazonic acid in maize. Food Addit Contam 28: 198-208.

23. Horn BW, Dorner JW (1999) Regional differences in production of aflatoxin B_1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Appl Environ Microbiol 65: 1444-1449.

24. Ehrlich KC, Montalbano BG, Cotty PJ (2007) Analysis of single nucleotide polymorphisms in three genes shows evidence for genetic isolation of certain *Aspergillus flavus* vegetative compatibility groups. FEMS Microbiol Lett 268: 231-236.

25. McAlpin CE, Wicklow DT (2005) Culture media and sources of nitrogen promoting the formation of stromata and ascocarps in *Petromyces alliaceus* (*Aspergillus* section *Flavi*). Can J Microbiol 51: 765-771.

26. Horn BW, Ramirez-Prado JH, Carbone I (2009) Sexual reproduction and recombination in the aflatoxin-producing fungus *Aspergillus parasiticus*. Fungal Genet Biol 46: 169-175.

27. Horn BW, Dorner JW (1998) Soil populations of *Aspergillus* species from section *Flavi* along a transect through peanut-growing regions of the United States. Mycologia 90: 767-776.

28. Utkhede RS, Rahe JE (1979) Wet-sieving floatation technique for isolation of sclerotia of *Sclerotium cepivorum* from muck soil. Phytopathology 69: 295-297.

29. Horn BW, Dorner JW (2009) Effect of nontoxigenic *Aspergillus flavus* and *A. parasiticus* on aflatoxin contamination of wounded peanut seeds inoculated with agricultural soil containing natural fungal populations. Biocont Sci Technol 19: 249-262.

30. Carbone I, Jakobek JL, Ramirez-Prado JH, Horn BW (2007) Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. Mol Ecol 16: 4401-4417.

31. Grubisha LC, Cotty PJ (2009) Twenty-four microsatellite markers for the aflatoxin-producing fungus *Aspergillus flavus*. Mol Ecol Resour 9: 264-267.

32. Aylor DL, Price EW, Carbone I (2006) SNAP: Combine and Map modules for multilocus population genetic analysis. Bioinformatics 22: 1399-1401.

33. Price EW, Carbone I (2005) SNAP: workbench management tool for evolutionary population genetic analysis. Bioinformatics 21: 402-404.

34. Monacell JT, Carbone I (2014) Mobyle SNAP Workbench: a web-based analysis portal for population genetics and evolutionary genomics. Bioinformatics 30: 1488-1490.

35. Nieuwenhuis BPS, Aanen DK (2012) Sexual selection in fungi. J Evol Biol 25: 2397-2411.

36. Holz G, Coertze S, Williamson B (2007) The ecology of *Botrytis* on plant surfaces. In: Elad Y, Williamson B, Tudzynski P, Delen N (eds) *Botrytis*: biology, pathology and control. Springer, Dordrecht, The Netherlands, 9-27.

37. Fukumori Y, Nakajima M, Akutsu K (2004) Microconidia act the role as spermatia in the sexual reproduction of *Botrytis cinerea*. J Gen Plant Pathol 70: 256-260.

38. Tadych M, Bergen MS, White Jr JF (2014) *Epichloë* spp. associated with grasses: new insights on life cycles, dissemination and evolution. Mycologia 106: 181-201.

39. Dyer PS, O'Gorman CM (2012) Sexual development and cryptic sexuality in fungi: insights from *Aspergillus* species. FEMS Microbiol Rev 36: 165-192.

40. Leslie JF, Raju NB (1985) Recessive mutations from natural populations of *Neurospora crassa* that are expressed in the sexual diplophase. Genetics 111: 759-777.

41. Raju NB (1992) Genetic control of the sexual cycle in *Neurospora*. Mycol Res 96: 241-262.

42. Xu J (2002) Estimating the spontaneous mutation rate of loss of sex in the human pathogenic fungus *Cryptococcus neoformans*. Genetics 162: 1157-1167.

43. Saleh D, Milazzo J, Adreit H, Tharreau D, Fournier E (2012) Asexual reproduction induces a rapid and permanent loss of sexual reproduction capacity in the rice fungal pathogen *Magnaporthe oryzae*: results of *in vitro* experimental evolution assays. BMC Evol Biol 12: 42.

44. Grubisha LC, Cotty PJ (2010) Genetic isolation among sympatric vegetative compatibility groups of the aflatoxin-producing fungus *Aspergillus flavus*. Mol Ecol 19: 269-280.

45. Benjamin CR (1955) Ascocarps of *Aspergillus* and *Penicillium*. Mycologia 47: 669-687.

46. Fennell DI, Warcup JH (1959) The ascocarps of *Aspergillus alliaceus*. Mycologia 51: 409-415.

47. Rudolph ED (1962) The effect of some physiological and environmental factors on sclerotial Aspergilli. Am J Bot 49: 71-78.

48. Leal JA, Gil R (1982) Ultrastructure of sclerotia of *Aspergillus alliaceus*. Trans Br Mycol Soc 78: 323-329.

49. Tewari JP (1983) Stromatic cell autolysis in *Petromyces alliaceus* during ascocarp formation. Trans Br Mycol Soc 80: 127-130.

50. Bojović-Cvetić D, Vujičić R (1988) Polysaccharide cytochemistry in maturing *Aspergillus flavus* sclerotia. Trans Br Mycol Soc 91: 619-624.

51. Wada R, Jin FJ, Koyama Y, Maruyama J, Kitamoto K (2014) Efficient formation of heterokaryotic sclerotia in the filamentous fungus *Aspergillus oryzae*. Appl Microbiol Biotechnol 98: 325-334.

52. Chang P-K, Ehrlich KC (2010) What does genetic diversity of *Aspergillus flavus* tell us about *Aspergillus oryzae*? Int J Food Microbiol 138: 189-199.

53. Zeng J, Feng S, Cai J, Wang L, Lin F, Pan Q (2009) Distribution of mating type and sexual status in Chinese rice blast populations. Plant Dis 93: 238-242.

54. Whittle CA, Johannesson H (2013) Evolutionary dynamics of sex-biased genes in a hermaphrodite fungus. Mol Biol Evol 30: 2435-2446.

55. Wilson AJ, Xu J (2012) Mitochondrial inheritance: diverse patterns and mechanisms with an emphasis on fungi. Mycology 3: 158-166.

56. Sweany RR, Damann Jr KE, Kaller M.D (2011) Comparison of soil and corn kernel *Aspergillus flavus* populations: evidence for niche specialization. Phytopathology 101: 952-95

Table 2.1 Sexual reproduction in *A. flavus* sclerotia from single strains of one mating type when incubated on sterile soil and on soil containing natural fungal populations.

Sclerotium-producing strain[a]			Sterile soil		Nonsterile soil		
MAT1-1	*MAT1-2*	VCG	No. sclerotia examined	% fertile sclerotia[b]	No. sclerotia examined	% fertile sclerotia[b]	No. progeny[c]
29507		33	300	0	300	58.7 ± 10.7[d]	36
29473		17	300	0	300	56.0 ± 2.6[d]	36
29537		63	300	0	718	0.1 ± 0.2[e]	12
	29536	62	300	0	312	80.2 ± 8.0	36
	21882	24	300	0	688	0.4 ± 0.8[e]	36
	29487	25	300	0	673	0[e]	
	AF36	YV36	300	0	674	0[e]	

[a]Strain numbers (except AF36) from Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois. Nonaflatoxigenic biocontrol strains include NRRL 21882 (from Afla-Guard) and AF36 (= NRRL 18543); all other strains produce aflatoxin B_1 and cyclopiazonic acid [5]. Mating-type designations from Ramirez-Prado et al. [12] and vegetative compatibility groups (VCGs) based on Horn and Greene [4] except for YV36 [24].

[b]Sclerotia with ascospores considered fertile; means ± SD based on three soil cups.

[c]Progeny obtained from three ascocarps in separate sclerotia (n = 12 progeny per ascocarp) except for NRRL 29537, which produced a single ascocarp.

[d,e]Pairwise chi-square test of independence failed to differentiate the fertility of these isolates ($P > 0.05$) (S1 Text).

Table 2.2 Sexual reproduction in single-strain and fertilized sclerotia of *A. flavus* under field conditions.

Strain(s)[a]			Field A			Field B			Field C	
			No. sclerotia examined[b]	No. fertile sclerotia[c]	No. progeny[d]	No. sclerotia examined[b]	No. fertile sclerotia[c]	No. progeny[d]	No. sclerotia examined[b]	No. fertile sclerotia[c]	No. progeny[d]
MAT1-1		*MAT1-2*									
Single-strain sclerotia											
29507			624 (74.3)	0		673 (88.9)	8 (1.2)	108	230 (96.2)	0	
29473			582 (69.3)	0		342 (92.0)	2 (0.6)	59	225 (95.2)	0	
29537			631 (68.0)	0		540 (56.0)	1 (0.2)	1	444 (42.7)	0	
		29536	407 (90.5)	0		281 (80.3)	0		423 (64.2)	0	
		21882	1273 (38.7)	0		917 (77.6)	0		671 (81.3)	0	
		29487	761 (84.4)	0		219 (73.8)	0		509 (98.7)	0	
		AF36	1117 (69.3)	0		517 (64.0)	0		414 (86.0)	0	
Fertilized sclerotia from crosses											
29473	×	29487	762 (88.0)	3 (0.4)	36	988 (84.0)	1 (0.1)	12	356 (94.8)	2 (0.6)	24
29537	×	29536	108 (45.2)	0		254 (38.2)	0		173 (59.0)	0	
29507	×	AF36	660 (33.3)	8 (1.2)	36	475 (36.0)	8 (1.7)	36	382 (18.7)	11 (2.9)	36
29473	×	AF36	875 (65.3)	79 (9.0)	36	523 (72.0)	17 (3.3)	36	198 (83.3)	8 (4.0)	36

Table 2.2 (continued)

Cross[a]									
29507 × 21882	442 (96.0)	49 (11.1)	36	581 (77.3)	36 (6.2)	36	317 (84.0)	72 (22.7)	36
29473 × 21882	514 (62.7)	0		343 (64.0)	2 (0.6)	24	288 (60.9)	3 (1.0)	36

[a]Strain numbers (except AF36) from Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois; mating-type designations from Ramirez-Prado et al. [12]; vegetative compatibility group designations are shown in Table 1. Biocontrol strains include NRRL 21882 (from Afla-Guard) and AF36 (= NRRL 18543).

[b]Percentage viability of sclerotia (n = 49-75) in parentheses.

[c]Sclerotia with ascospore-bearing ascocarps considered fertile; percentage of sclerotia that were fertile in parentheses.

[d]In single-strain sclerotia from Field B, variable numbers of progeny were obtained from ascocarps in all available fertile sclerotia; in fertilized sclerotia from crosses in Fields A-C, progeny were obtained from 1-3 ascocarps in separate sclerotia (n = 12 progeny per ascocarp).

Table 2.3 Reciprocal crosses in *A. flavus* in which single-strain sclerotia were incubated on sterilized soil inoculated with conidia.

Sclerotia on soil[a]			Conidia in sterilized soil[a]				
MAT1-1	*MAT1-2*		*MAT1-1*	*MAT1-2*	No. sclerotia examined	% fertile sclerotia[b]	No. progeny[c]
29537		×		29536	480	1.1 ± 0.9^{d}	36
	29536	×	29537		300	98.7 ± 0.6^{d}	36
29537		×	29507		300	0	
	29536	×		AF36	300	0	
29507		×		21882	300	96.7 ± 2.3^{e}	35
	21882	×	29507		300	1.0 ± 0.0^{e}	36
29507		×	29473		300	0	
	21882	×		29536	300	0	
29473		×		AF36	300	85.7 ± 0.6^{f}	36
	AF36	×	29473		300	27.7 ± 12.5^{f}	36
29473		×	29537		300	0	
	AF36	×		21882	300	0	

[a]Strain numbers (except AF36) from Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois; mating-type designations from Ramirez-Prado et al. [12]; vegetative compatibility group designations are shown in Table 1. Biocontrol strains include NRRL 21882 (from Afla-Guard) and AF36 (= NRRL 18543).

[b]Sclerotia with ascospores considered fertile; means ± SD based on three soil cups.

[c]Progeny obtained from three ascocarps in separate sclerotia (n = 11-12 progeny per ascocarp).

[d,e,f]Reciprocal crosses within each pair significantly different ($P < 0.0001$) according to chi-square test of independence (S1 Text).

Table 2.4 Sexual reproduction in *A. flavus* sclerotia obtained from crosses and incubated under laboratory conditions.

Cross[a]			Culture slants (14 d)[b]		Culture slants (4 mo)		Nonsterile soil cups (4 mo)	
MAT1-1		*MAT1-2*	No. sclerotia examined	% fertile sclerotia	No. sclerotia examined	% fertile sclerotia[c]	No. sclerotia examined	% fertile sclerotia[c]
29473	×	29487	1050	0	600	14.7 ± 5.6	240	42.8 ± 8.6
29537	×	29536	851	0	600	50.5 ± 8.4[d]	240	61.1 ± 23.5
29507	×	AF36	1004	0	499	91.5 ± 3.8	240	78.0 ± 23.5[e]
29473	×	AF36	750	0	576	76.0 ± 5.5	240	71.0 ± 14.6
29507	×	21882	1050	0	600	54.5 ± 12.8[d]	210	78.3 ± 3.4[e]
29473	×	21882	1002	0	600	24.8 ± 21.0	240	24.9 ± 10.5

[a]Strain numbers (except AF36) from the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois; AF36 (= NRRL 18543) and NRRL 21882 are nonaflatoxigenic biocontrol strains.

[b]Sclerotia harvested for adding to nonsterile soil cups (shown in this table) and to Fields A-C (Table 2).

[c]Sclerotia with ascospores considered fertile; means ± SD based on six MCA slants or soil cups.

[d,e]Pairwise chi-square test of independence failed to differentiate the fertility of these isolates; all other values were significantly different ($P < 0.01$) (S1 Text).

Table 2.5 Genotype data for *A. flavus* progeny from single-strain sclerotia fertilized by strains from natural soil populations when incubated under laboratory conditions.

	Loci examined			
Strain[a]	Chr. 6 *MAT*	Chr. 2 *AF17*	Chr. 7 *AF48*	Chr. 3 *aflF/aflU*
Parents				
NRRL 29507	1	H1	H1	III
Wild strain A[b]	2	H2	H2	I
Wild strain B[b]	2	H3	H3	II
Wild strain C[b]	2	H4	H4	II
Progeny				
Ascocarp 1				
IC5956	2	H2	H1	III
IC5957	1	H1	H2	I
IC5958	1	H1	H1	III
Ascocarp 2				
IC5968	1	H3	H3	-[c]
IC5969	1	H3	H3	-[c]
IC5970	1	H1	H3	II
Ascocarp 3				
IC5980	2	H4	H4	II
IC5981	2	H1	H1	III
IC5982	2	H1	H1	III
Parents				
NRRL 29473	1	H1	H1	III
Wild strain A[b]	2	X[d]	H2	I
Wild strain B[b]	2	X[d]	H3	I
Wild strain C[b]	2	H4	X[d]	I
Progeny				
Ascocarp 1				
IC5151	2	H1	H2	I

Table 2.5 (continued)

IC5152	2	H1	H1	I
IC5154	1	H1	H1	-[c]
Ascocarp 2				
IC5164	1	H1	H3	III
IC5166	1&2	H1	H1	III/I[e]
IC5167	1	H1	H3	-[c]
Ascocarp 3				
IC5186	2	H1	H1	I
IC5188	1	H4	H1	I
IC5189	1	H1	H1	I
Parents				
NRRL 29536	2	H1	H1	III
Wild strain A[b]	1	H2	H2	I
Wild strain B[b]	1	H3	X[d]	II
Progeny				
Ascocarp 1[f]				
IC5197	1&2	H2	H2	III
IC5198	1	H2	H1	I
IC5201	1	H2	H2	III
Ascocarp 2				
IC5209	2	H3	H1	III
IC5210	2	H1	H1	III
IC5211	1&2	H1	H1	II
Ascocarp 3[f]				
IC5221	1	H2	H2	I
IC5222	1	H2	H2	I
IC5223	2	H2	H1	III

[a]NRRL strain numbers from the Agricultural Research Service Culture Collection, Peoria, Illinois, and IC strain numbers from the Ignazio Carbone Culture Collection, North Carolina State University, Raleigh, North Carolina.

[b]MLSTs of wild strains determined by identification of alleles in progeny differing from those of the known sclerotial strain.

[c]PCR did not amplify.

[d]Allele not seen in tested progeny.

[e]PCR yielded two amplicons corresponding to deletion types III and I.

[f]Ascocarps 1 and 3 fertilized by wild strain with the same MLST.

Table 2.6 Soil populations of *Aspergillus* section *Flavi* species (CFU/g) in fields prior to application of *A. flavus* sclerotia.[a]

Species	Field A	Field B	Field C
A. flavus L strain	398 ± 230	8135 ± 1884 [b]	391 ± 173
A. flavus S strain	20 ± 30	136 ± 111 [b]	40 ± 21
A. parasiticus	83 ± 48	179 ± 68	255 ± 375
A. tamarii	0	4 ± 8	260 ± 183 [b]
A. caelatus	4 ± 5 [b]	18 ± 13 [b]	654 ± 164 [b]
A. alliaceus	1025 ± 788 [b]	7 ± 10	13 ± 15

[a]Means ± SD based on five soil samples.

[b]Density of the species or strain significantly different ($P < 0.05$) from the other fields based on ANOVA and SNK comparison of means.

Table 2.7 Genotype data for *A. flavus* progeny from single-strain sclerotia fertilized by strains from natural soil populations in the field.

	Loci examined			
	Chr. 6	Chr. 2	Chr. 7	Chr. 3
Strain[a]	*MAT*	*AF17*	*AF48*	*aflF/aflU*
Parents				
NRRL 29507	1	H1	H1	III
Wild strain A[b]	2	H2	H2	II
Wild strain B[b]	2	H3	H3	II
Wild strain C[b]	2	H4	H4	II
Progeny				
Ascocarp 1				
IC9156	2	H2	H2	III
IC9157	1	H1	H2	II
IC9158	1	H2	H1	III
Ascocarp 2				
IC9168	2	H3	H3	III
IC9169	1	H1	H1	III
IC9170	1	H1	H1	II
Ascocarp 3				
IC9180	1	H1	H4	III
IC9181	2	H1	H1	II
IC9182	2	H4	H4	III
Parents				
NRRL 29473	1	H1	H1	III
Wild strain A[b]	2	H2	H2	II
Wild strain B[b]	2	X[c]	X[c]	II
Progeny				
Ascocarp 1				
IC10107	2	H2	H2	III
IC10108	1	H1	H1	II

Table 2.7 (continued)

IC10109	2	H2	H1	III
Ascocarp 2A[d]				
IC10119	1&2	H1	H1	II
IC10120	1&2	H1	H1	III
IC10123	2	H1	H1	II
Ascocarp 2B[d]				
IC10148	1&2	H1	H1	III
IC10149	1	H1	H1	III
IC10152	1	H1	H1	II

[a]NRRL strain numbers from the Agricultural Research Service Culture Collection, Peoria, Illinois, and IC strain numbers from the Ignazio Carbone Culture Collection, North Carolina State University, Raleigh, North Carolina.

[b]MLSTs of wild strains determined by identification of alleles in progeny differing from those of the known sclerotial strain.

[c]Allele not seen in tested progeny.

[d]Ascocarps 2A and 2B from the same sclerotium; fertilized by wild strain with the same MLST.

Table 2.8 Genotype data for *A. flavus* progeny obtained from fertilized sclerotia that were applied to fields before ascocarp formation.

	Loci examined			
	Chr. 6	Chr. 2	Chr. 7	Chr. 3
Strain[a]	*MAT*	*AF17*	*AF48*	*aflF/aflU*
Parents				
NRRL 29507	1	H1	H1	H1
AF36	2	H2	H2[b]	H2
Progeny				
Field A				
Ascocarp 1				
IC9312	1	H2	H2[b]	H1
IC9313	1	H1	H1	-[c]
IC9316	1	H2	H1	-[c]
Ascocarp 2				
IC9324	2	H2	H1	H2
IC9326	2	H2	H2[b]	H2
IC9327	2	H1	H1	H1
Field B				
Ascocarp 1				
IC9452	1	H1	H2[b]	H2
IC9453	2	H1	H1	H2
IC9455	1	H2	H2[b]	H2
Ascocarp 2				
IC9464	2	H2	H2[b]	H2
IC9467	2	H1	H1	H1
IC9468	1	H1	H1	H2
Field C				
Ascocarp 1				
IC10222	2	H1	H2[b]	H2
IC10223	1	H1	H2[b]	H1

Table 2.8 (continued)

IC10226	1	H1	H2[b]	H2
Ascocarp 2				
IC10235	1	H1	H2[b]	H1
IC10236	1	H1	H2[b]	H2
IC10237	2	H1	H2[b]	H1
Parents				
NRRL 29473	1	H1	H1	H1
AF36	2	H2	H2[b]	H2
Progeny				
Field A				
Ascocarp 1				
IC9348	2	H1	H2[b]	H1
IC9349	2	H1	H2[b]	H2
IC9350	1	H1	H2[b]	H1
Ascocarp 2				
IC9360	2	H1	H2[b]	H2
IC9361	1	H1	H2[b]	H1
IC9362	1	H1	H1	H1
Field B				
Ascocarp 1				
IC9488	1	H1	H2[b]	H1
IC9489	1	H1	H1	H1
IC9490	2	H1	H2[b]	H2
Ascocarp 2				
IC9512	2	H1	H1	H1
IC9513	2	H1	H2[b]	H2
IC9514	1	H1	H2[b]	H2
Field C				
Ascocarp 1				
IC10258	1	H1	H1	H2
IC10259	2	H1	H2[b]	H2
IC10260	2	H1	H1	H2

Table 2.8 (continued)

Ascocarp 2				
IC10270	2	H1	H1	H2
IC10271	2	H1	H2[b]	H2
IC10279	2	H1	H2[b]	H2
Parents				
NRRL 29507	1	H1	H1	H1
NRRL 21882	2	H2	H2	-[d]
Progeny				
Field A				
Ascocarp 1				
IC9384	1	H2	H2	H1
IC9385	2	H2	H1	H1
IC9386	1	H2	H2	-[d]
Ascocarp 2				
IC9396	2	H2	H2	-[d]
IC9397	2	H2	H1	H1
IC9398	2	H2	H1	H1
Field B				
Ascocarp 1				
IC9524	1	H2	H1	-[d]
IC9525	2	H2	H2	H1
IC9526	2	H2	H1	H1
Ascocarp 2				
IC9536	1	H1	H2	H1
IC9537	2	H1	H2	H1
IC9538	1	H2	H2	-[d]
Field C				
Ascocarp 1				
IC10294	1	H1	H1	H1
IC10295	1	H1	H2	-[d]
IC10296	1	H2	H2	-[d]
Ascocarp 2				
IC10306	2	H2	H1	-[d]

Table 2.8 (continued)

IC10307	1	H2	H1	H1
IC10308	2	H2	H2	H1

[a]NRRL strain numbers from the Agricultural Research Service Culture Collection, Peoria, Illinois, and IC strain numbers from the Ignazio Carbone Culture Collection, North Carolina State University, Raleigh, North Carolina.

[b]Due to the high number of triplets at the *AF48* locus in AF36, sequenced products range from 66 to 70 GAA repeats.

[c]PCR did not amplify.

[d]NRRL 21882 has a deletion of the entire aflatoxin gene cluster [11], which accounts for missing *aflC* in some of the progeny.

Table 2.9 Nuclear and mitochondrial loci for progeny from reciprocal crosses between single-strain sclerotia and conidia inoculated in sterile soil.

	Loci examined	
Strain[a]	Chr. 6 *MAT*	Mitochondria *AF-MIT-1*
Parents		
NRRL 29537 (sclerotia)	1	H1
NRRL 29536 (conidia)	2	H2
Progeny		
Ascocarp 1		
IC5813	1	H1
IC5814	2	H1
Ascocarp 2		
IC5825	1&2	H1
IC5826	2	H1
Ascocarp 3		
IC5837	2	H1
IC5838	1	H1
Parents		
NRRL 29537 (conidia)	1	H1
NRRL 29536 (sclerotia)	2	H2
Progeny		
Ascocarp 1		
IC5849	1	H2
IC5850	1&2	H2
Ascocarp 2		
IC5861	2	H2
IC5862	1&2	H2
Ascocarp 3		
IC5873	2	H2
IC5874	2	H2

Table 2.9 (continued)

Strain[a]	Chr. 6 *MAT*	Mitochondria *AF-MIT-3*
Parents		
NRRL 29507 (sclerotia)	1	H1
NRRL 21882 (conidia)	2	H2
Progeny		
Ascocarp 1		
IC5885	2	H1
IC5886	2	H1
Ascocarp 2		
IC5897	1&2	H1
IC5898	2	-[b]
Ascocarp 3		
IC5909	1&2	H1
IC5910	2	H1
Parents		
NRRL 29507(conidia)	1	H1
NRRL 21882 (sclerotia)	2	H2
Progeny		
Ascocarp 1		
IC5920	2	H2
IC5921	2	H2
Ascocarp 2		
IC5932	2	H2
IC5933	2	H2
Ascocarp 3		
IC5944	1	H2
IC5945	1&2	H2

Table 2.9 (continued)

Strain[a]	Chr. 6 *MAT*	Mitochondria *AF-MIT-4*
Parents		
NRRL 29473 (sclerotia)	1	H1
AF36 (conidia)	2	H2
Progeny		
Ascocarp 1		
IC5741	2	H1
IC5742	1	H1
Ascocarp 2		
IC5753	2	H1
IC5754	1	H1
Ascocarp 3		
IC5765	2	H1
IC5766	2	H1
Parents		
NRRL 29473 (conidia)	1	H1
AF36 (sclerotia)	2	H2
Progeny		
Ascocarp 1		
IC5777	1	H2
IC5778	1&2	H2
Ascocarp 2		
IC5789	1	H2
IC5790	1&2	H2
Ascocarp 3		
IC5801	1	H2
IC5802	2	H2

[a]NRRL strain numbers from the Agricultural Research Service Culture Collection, Peoria, Illinois, and IC strain numbers from the Ignazio Carbone Culture Collection, North Carolina State University, Raleigh, North Carolina.

[b]PCR did not amplify.

Table 2.S1 Weather conditions at three fields (2013-2014) where single-strain and fertilized sclerotia of *A. flavus* were applied.

	Air temperature (°C)[a]			Rainfall (cm)[b]		
Month	Minimum	Maximum	Mean	Field A	Field B	Field C
2013						
Apr 16-30	12.6 ± 0.2	25.9 ± 0.3	18.8 ± 0.1	3.8 (3)	3.6 (3)	6.7 (2)
May	15.2 ± 0.1	28.0 ± 0.6	21.5 ± 0.3	2.8 (5)	4.6 (5)	3.0 (4)
Jun	20.8 ± 0.3	31.5 ± 0.5	25.2 ± 0.2	25.4 (14)	15.5 (13)	16.2 (15)
Jul	21.1 ± 0.5	30.8 ± 0.5	24.6 ± 0.3	20.9 (21)	20.2 (21)	26.2 (18)
Aug	20.8 ± 0.6	31.5 ± 0.5	25.2 ± 0.3	15.7 (8)	17.6 (8)	20.0 (8)
Sep	19.0 ± 0.4	30.0 ± 0.4	23.6 ± 0.3	5.8 (7)	5.5 (5)	7.1 (8)
Oct	13.4 ± 0.5	26.1 ± 0.4	19.1 ± 0.3	3.9 (2)	3.0 (2)	7.8 (1)
Nov	7.1 ± 0.3	19.0 ± 0.2	12.5 ± 0.2	7.1 (4)	9.9 (4)	7.2 (3)
Dec	6.3 ± 0.1	17.6 ± 0.2	11.5 ± 0.1	19.5 (10)	18.6 (10)	19.6 (9)
2014						
Jan	-0.8 ± 0.3	11.8 ± 0.1	5.1 ± 0.2	7.0 (11)	8.1 (12)	10.7 (11)
Feb	4.9 ± 0.3	17.1 ± 0.2	10.8 ± 0.1	11.9 (9)	13.4 (9)	14.8 (9)
Mar	6.1 ± 0.1	19.4 ± 0.3	12.7 ± 0.1	10.0 (8)	11.9 (8)	12.5 (8)
Apr 1-15	11.3 ± 0.4	24.5 ± 0.4	17.8 ± 0.2	17.6 (9)	14.1 (7)	15.2 (4)

[a]Means ± SD (n = 3) based on monthly means of three fields; temperatures for each field were calculated from daily temperatures for each month (n = 28-31 days), except for April (n = 15).

[b]Numbers in parentheses are number of days per month in which rainfall exceeded 1 mm

SUPPLEMENTAL TEXT

Table 1

Total number of fertile sclerotia and unfertile sclerotia for each sclerotium-producing strain was calculated by summing the replicates. Chi-square test of independence was run using Microsoft Excel 2013 CHISQ.TEST function for pairwise comparisons of fertility between sclerotium-producing strains. Comparisons with $P < 0.05$ were considered to show significant differences in fertility.

	NRRL 29507	NRRL 29473	NRRL 29537	NRRL 29536	NRRL 21882	NRRL 29487	AF36
NRRL 29507	-	0.51	9.51E-112	4.47E-09	7.50E-106	7.78E-107	5.74E-107
NRRL 29473	-	-	1.02E-105	7.66E-11	5.22E-100	4.51E-101	3.37E-101
NRRL 29537	-	-	-	4.33E-167	0.30	0.33	0.33
NRRL 29536	-	-	-	-	9.66E-160	5.00E-160	3.31E-160
NRRL 21882	-	-	-	-	-	0.09	0.09
NRRL 29487	-	-	-	-	-	-	1.00*
AF36	-	-	-	-	-	-	-

*Comparison between AF36 and NRRL 29487 performed with rxc XML on Mobyle SNAP Workbench [34].

Table 2

We tested using a hypergeometric test whether we would expect to see the number of fertile sclerotia in Field B if the three fields (A, B and C) were equally likely to produce fertile sclerotia. The hypergeometric distribution models the probability of i successes in a sample of k given a population of size N with n successes and m failures. If all three fields are identical, this model represents the probability of a field (k) producing a number of fertile single-strain sclerotia (i). Each application of single-strain sclerotia represents an individual in the population ($N = 21$). Applications that produced any fertilized sclerotia are successes ($n = 3$) while applications in which fertile sclerotia were not found are failures ($m = 18$). The probability of one field ($k = 7$) containing all 3 fertile sclerotia applications ($i = 3$) is $P = 0.03$, calculated using Microsoft Excel 2013 HYPGEOM.DIST function.

Table 3

Total number of fertile sclerotia and unfertile sclerotia for each sclerotial strain × conidial strain cross was calculated by summing the replicates. Chi-square test of independence was run using Microsoft Excel 2013 CHISQ.TEST function for pairwise comparisons of fertility within reciprocal crosses. Comparisons with $P < 0.05$ were considered to show significant differences in fertility.

MAT1-1 sclerotial strain x *MAT1-2* conidial strain	*MAT1-2* sclerotial strain x *MAT1-1* conidial strain	*P*-value
NRRL 29537 × NRRL 29536	NRRL 29536 × NRRL 29537	1.34E-162
NRRL 29473 × AF36	AF36 × NRRL 29473	3.81E-24
NRRL 29507 × NRRL 21882	NRRL 21882 × NRRL 29507	1.07E-61

Table 4

Total number of fertile sclerotia and unfertile sclerotia for each cross was calculated by summing the replicates. Chi-square test of independence was run using Microsoft Excel 2013 CHISQ.TEST function for pairwise comparisons of fertility between crosses in which sclerotia were incubated for 4 mo in culture slants and in nonsterile soil cups. Comparisons with $P < 0.05$ were considered to show significant differences in fertility.

	Culture slants (4 mo)					
	NRRL 29473 × NRRL 29487	NRRL 29537 × NRRL 29536	NRRL 29507 × AF36	NRRL 29473 × AF36	NRRL 29507 × NRRL 21882	NRRL 29473 × NRRL 21882
NRRL 29473 × NRRL 29487	-	4.99E-40	2.03E-144	6.83E-99	1.12E-47	9.73E-06
NRRL 29537 × NRRL 29536	-	-	2.01E-50	2.13E-19	0.17	4.53E-20
NRRL 29507 × AF36	-	-	-	7.27E-13	2.76E-43	8.81E-111
NRRL 29473 × AF36	-	-	-	-	1.58E-14	1.43E-68
NRRL 29507 × NRRL 21882	-	-	-	-	-	8.32E-26
NRRL 29473 × NRRL 21882	-	-	-	-	-	-

Nonsterile soil cups (4 mo)

	NRRL 29473 × NRRL 29487	NRRL 29537 × NRRL 29536	NRRL 29507 × AF36	NRRL 29473 × AF36	NRRL 29507 × NRRL 21882	NRRL 29473 × NRRL 21882
NRRL 29473 × NRRL 29487	-	0.0004	9.54E-19	2.90E-10	9.14E-16	1.69E-05
NRRL 29537 × NRRL 29536	-	-	3.88E-08	0.005	1.68E-06	1.02E-14
NRRL 29507 × AF36	-	-	-	0.006	0.38	1.09E-36
NRRL 29473 × AF36	-	-	-	-	0.03	5.89E-25
NRRL 29507 × NRRL 21882	-	-	-	-	-	1.95E-29
NRRL 29473 × NRRL 21882	-	-	-	-	-	-

CHAPTER 3

HPLC Quantitation of Aflatoxin B_1 from Fungal Mycelium Culture

Published by Journal of Microbiological Methods

Richard M. Gell [1,*], and Ignazio Carbone [1]

[1]Center for Integrated Fungal Research, Program of Genetics, Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA.

*Correspondence: rmgell@ncsu.edu; Tel.: +919-513-4867

CONTRIBUTION SUMMARY

For the experiments in this paper, I developed the protocol for aflatoxin extraction from media that was consistently repeatable in our lab from guidance given by Dr. Victor Sobolev (National Peanut Research Laboratory, USDA-ARS, Dawson, GA). I designed and performed the aflatoxin standards extraction section and assisted with the aflatoxin extractions from populations, which were performed by Vicki Cornish and Hafsa Hurshe. I wrote and edited the manuscript with Dr. Ignazio Carbone.

ABSTRACT

Aflatoxins are mycotoxins that contaminate agricultural products when infected by toxigenic *Aspergillus flavus*. Methods for quantifying aflatoxin from culture using chromatography are available but are not optimized for population studies. We provide details of a method for preparation and quantitation of aflatoxin B_1 from fungal cultures that satisfy those needs.

Keywords: *Aspergillus flavus*; aflatoxin B_1; HPLC; mycotoxins

INTRODUCTION

Aflatoxins are carcinogenic secondary metabolites produced by *Aspergillus flavus* and allied species that contaminate agricultural commodities [1,2]. Aflatoxins are known to accumulate in fungal mycelia, spores and sclerotia, and are also excreted into their environment [3,4]. Of the four aflatoxin metabolites (B_1, B_2, G_1, G_2) that can potentially accumulate in these fungi, aflatoxin B_1 is the most carcinogenic and most abundantly produced. Due to its toxicity to animals and humans the United States Food and Drug Administration (FDA) tightly regulates acceptable levels of aflatoxin B_1 in food products for human consumption at less than 20 ppb and animal feed at less than 20 ppb to less than 300 ppb depending on crop, livestock, and animal's maturity [5,6]. These analyses are based on aflatoxin found in the grain or feedstuff, which could be derived from several aflatoxigenic fungi. Commercially available methods for detection and determination of aflatoxin levels based on antibodies or enzyme-linked immunosorbent assay (ELISA) are designed to extract and measure aflatoxins from plant derived substrates, at levels as low as 0.5 ppb to levels higher than 100 ppb [7,8]. However, these approaches do not indicate if one or multiple strains or species are producing the toxins nor do they distinguish between the different aflatoxin chemotypes of those strains.

While this information may not be critical for regulatory purposes, understanding the species and lineage composition of strains that are aflatoxigenic and non-aflatoxigenic as well as the relative amounts of B_1 aflatoxin produced between aflatoxigenic strains is important to population genetic studies with the ability to improve agricultural management strategies [9]. Increasing evidence exists that balancing selection is maintaining fungal lineages that are aflatoxigenic and non-aflatoxigenic in natural populations [10-13]. A better understanding of the aflatoxin producing potential of strains in these lineages is important when evaluating the

population genetic consequences of management strategies that deploy non-aflatoxigenic strains as biological control agents [14,15].

Previous approaches to aflatoxin quantification for research explored extractions directly from fungal mycelium grown in culture and then with aflatoxins found in agricultural products (e.g. oil-seeds and kernels) using simple chemical cleanup columns [16,17]. To further facilitate quantitation of aflatoxin production across isolates in a fast but increasingly reliable approach and provide a detailed methodology for aflatoxin quantitation needed by the community, we adapted these methodologies using a HPLC method that consistently quantifies aflatoxin B_1 in the media of fungal mycelium cultures after extraction with chloroform and purification by chemical cleanup columns. Obtaining aflatoxin concentrations only from culture media reduces the problematic non-aflatoxin signals during HPLC increasing our ability to replicate previous approaches and permits relevant quantitation from isolates that are non-aflatoxigenic and those that produce aflatoxin at low levels; this is important because even very low doses (20 ng/ml) of aflatoxin B_1 can be toxic to humans and animals [18].

MATERIALS AND METHODS

Solid and Liquid Fungal Cultures for Aflatoxin Analysis

Cultures for aflatoxin testing were grown on PDA plates for 7 days (5-dark, 2-light) at 30°C, which is sufficient for yielding abundant sporulation. Following sporulation, liquid cultures were grown in 8mL of YES Media, a media containing 2% yeast extract and 20% sucrose known to facilitate the production of aflatoxins, [19] with 75 µg/mL ampicillin within a 16mL vial (ThermoSci Screw Vial Convenience Kit, Solid-top Cap from Thermo Scientific, B7800-4). A 1 µL inoculating loop (VWR 12000-808) was used to inoculate by touching the surface of the strain's plate in a region with conidia, obtaining a ball of conidia approximately the size of the hole in the inoculating loop, and then inserting it into a YES vial by touching the loop to the media and gently agitating. Caps were screwed onto each vial by closing completely and then half turn backward to allow for airflow. Vials were sealed by a single wrap of parafilm to secure and prevent loss of volume due to dehydration without impacting oxygen permeability and incubated under constant light at 30°C for 7-days. Three liquid cultures were produced as replicates for each strain tested.

Aflatoxin Extractions

In a biosafety cabinet, 1mL of each fully incubated culture was taken and transferred to a 4mL vial (ThermoSci Screw Vial Convenience Kit, Solid-top Cap from Thermo Scientific, B7800-2). The culture samples were obtained from just below the mat of mycelium while minimizing the amount of detritus transferred.

In fume hood, 1mL of Chloroform HPLC Grade, Alfa Aesar (VWR AA43685-K2) was added to each culture being extracted, and vortexed vigorously for 10 seconds. Samples were left at rest for 30 minutes to allow for separation of layers; if any emulsive cultures remained, they

were centrifuged for 2 minutes at 3,000 g. A 1mL pipetman was used to transfer 0.5mL of the chloroform layer to a new 4mL vial minimizing any media or tissue debris contaminating the sample. Each extraction was placed in a heated bead bath under a low-pressure stream of UHP GR 5.0 Nitrogen until chloroform completely evaporated. Caps were resealed rapidly after removing the nitrogen stream to limit oxygen exposure. Extractions were then stored sealed and dry at 4°C in the dark.

Extract Purification

One milliliter of methanol (HiPerSolv CHROMANORM® gradient for HPLC; VWR BDH20864.100E) was added to each dried sample and suspended by agitating. The suspended samples were passed through 1mL polypropylene SPE tubes (Sigma-Aldrich, 57023), containing 200µL of alumina basic, 60-235 mesh (Fisher, A941-500) between PE frits, 20µm porosity (Sigma-Aldrich, 57023) into new 4mL vials as the purified sample. The bottom tips of clean-up columns each containing trace amounts of its purified sample were placed under long-wave UV, which causes aflatoxin to fluoresce. The fluorescence of samples' clean-up columns was compared to previous results to determine dilution amounts needed to allow for only one injection. Purified samples were stored stably at 4°C in the dark for 0-2 days before HPLC injection which allows for multiple sets of extractions to be analyzed at once.

HPLC

Aflatoxin quantifications were performed by the Biomanufacturing Training and Education Center's Bioprocess and Analytical Services at North Carolina State University following previously published specifications [20]. In summary, samples were analyzed by reversed phase HPLC on a Shimadzu Prominence system interfaced with Shimadzu Labsolutions Version 5.54 using a Phenomenex Kinetex, 2.6 µm, C18, 100 Å, 150 x 4.6 mm column and

fluorescence detection. Mobile phases consisted of deionized water (A) and methanol (B) filtered through a 0.22 mm membrane filter prior to use. A gradient program was utilized, with a starting condition of A:B (50:50, v/v) held for 11 minutes. The proportion of A:B was changed (5:95, v/v) over the course of 1 minute, and held for 5 minutes. The proportion of A:B was returned to initial conditions (50:50, v/v) over the course of 0.5 minutes and held for 7.5 minutes for a total run time of 25 minutes. Sample injection volume was 10 µL, with a flow rate of 0.5 mL/min, an excitation wavelength of 365 nm, an emission wavelength of 455 nm and a column temperature of 40°C. The working aflatoxin B_1 (Sigma-Aldrich A6636-5MG) standard curve was based on a five-point calibration curve for the range of 0.0625-1.0 µg/mL. Serial dilutions were used to determine the limit of aflatoxin B_1 detection at 0.0020 µg/mL and quantification at 0.0039 µg/mL.

Aflatoxin Recovery from Spiking

Aflatoxin B_1 (Sigma-Aldrich A6636-5MG) dissolved in methanol to a concentration of 1000 µg/mL was added to each sample at the appropriate volume; 1, 6, 20, and 50 µL respectively. Each IC201 and IC1179 spiking sample was processed as above until the subsampling 1mL of liquid culture step. Each spiked media sample contained only the 7mL of YES media with no introduced strain of *Aspergillus flavus*. For non-chloroform spiking samples, aflatoxins were added to the 1mL of culture after it was transferred to its new vial and vortexed, before adding chloroform and being processed as other samples. Each chloroform spiking sample contained 1 mL chloroform amended with the appropriate aflatoxin spiking amount followed by vortexing and processing of the top 0.5 mL of chloroform as the sample for drying under nitrogen and handling, as the other dried samples.

Known Aflatoxigenic Strains and Population Sample Isolates

Five strains of *A. flavus* previously shown to produce aflatoxins were retrieved and tested for aflatoxin concentration. These five strains native to Georgia were originally harvested from corn kernels (IC218, IC225), soil samples (IC229, IC278), and peanut seeds (IC308)[10].

Forty-two isolates obtained as part of an ongoing exploration of genetic structure in *A. flavus* field populations [21] were also tested for aflatoxin concentration with this methodology. These strains came from soil and kernel samples collected between 2013 and 2014, in Texas, North Carolina, Arkansas, and Indiana.

RESULTS AND DISCUSSION

Aflatoxin Recovery from Spiking

The percent recovery of aflatoxins with this method was determined by extraction and quantification of non-aflatoxigenic samples spiked with aflatoxin B_1 to achieve four different concentrations: 1, 6, 20, and 50 µg/mL of culture. To understand the source of any variation a variety of non-aflatoxigenic sample types were tested including media inoculated with *A. flavus* biocontrol strains Afla-Guard (=NRRL 21882, IC201) and AF36 (=NRRL 18543, IC1179) to examine the impact of any cellular debris and other secreted metabolites, media-only samples that were not inoculated with any strain to examine how well aflatoxin is transferred between the media and chloroform, and samples where aflatoxin was spiked directly into 1mL of chloroform to explore the amount of aflatoxin lost due to the resuspension and purification with the chemical cleanup column.

Recovery of aflatoxins under all experimental conditions and concentrations tested showed fairly consistent retention with an overall average percent recovery of 77.68% ± 9.52% (Table 1). There was no substantial variation between results observed under experimental conditions where the aflatoxins were removed from media, with or without non-aflatoxigenic cultures present, and results where aflatoxin B_1 was applied directly to the chloroform supporting the extraction method. The consistent loss, which was observed in all samples, appears to be during either resuspension or purification. Additional variations of these steps were not tested due to the risks of aflatoxin degradation with prolonged handling and excessive dilution of samples with low concentrations. Variations from this expected loss occurred across experimental conditions and concentrations providing no clear indications of what causes this phenomenon but in no case did this variance alter the magnitude or relative concentration of the

sample. None of the experimental conditions reported any aflatoxin when no aflatoxin was introduced.

Aflatoxin Recovery from Known Aflatoxigenic Strains

To initially assess the protocol's ability to quantitate aflatoxin from fungal mycelium culture, extractions were performed on five strains of *Aspergillus flavus* known to produce aflatoxins across a spectrum of concentrations [10]. Three replicate cultures were grown and aflatoxins extracted in parallel for each strain to reduce sample variance that could occur due to environmental variability while examining what variation remains due to growth differences and extraction variability.

Aflatoxin B_1 concentrations ranged from 0.08 μg/mL to 29.58 μg/mL across the control samples with the highest Relative Standard Deviation (RSD) calculated as 58.08 for a set of three replicates (Table 2). Several strains were low producers with aflatoxin concentrations less than 0.50 μg/mL. For most of the strains, the results showed proportionally larger deviations from their mean than was produced with the spiking experiment. This variation in aflatoxin B_1 production by strains has been reported previously and explained by genotype by environment effects [15,22,23]. Mean aflatoxin concentrations between samples were comparable and show noticeably distinct levels across the selected strains supporting this protocol's use as a simple method for aflatoxin quantitation across an *A. flavus* population's diverse phenotype range.

Aflatoxin Recovery from Population Sample Isolates

Aspergillus flavus isolates can be separated genetically into two lineages; lineage IC which are known to produce higher levels of aflatoxin B_1 and lineage IB which have more nontoxigenic isolates and even toxigenic isolates produce less aflatoxins[9,24,25]. Aflatoxin B_1 concentrations of the toxigenic isolates from the high producing lineage had a broader range

from averages of 0.06 µg/mL to 39.12 µg/mL and RSD averages 40.93% compared to those from the low aflatoxin producing lineage with averages of 0.02 µg/mL to 1.99 µg/mL and RSD averages 60.81% (Table S1). Ten of the isolates produced no aflatoxin from any of the three extractions performed for each, suggesting that these isolates are likely non-aflatoxin producers. Of these ten nontoxigenic, only two were from the high toxin producing lineage while eight were from the lineage expected to contain fewer toxigenic isolates. Only three of thirty-two aflatoxigenic isolates; IC6510, IC14611, and IC14677, have an extraction where aflatoxin was not detected, supporting the consistency of this methodology for the detection of aflatoxin from aflatoxigenic isolates.

CONCLUSIONS

We describe an HPLC method for quantitation of aflatoxin B_1 in the media of fungal mycelium cultures. The method is scalable and can determine relative aflatoxin producing potential across the spectrum of possible yields as shown by quantitation of aflatoxin B_1 from over forty isolates as part of a larger population genetics study.

This study focused on the results for aflatoxin B_1, the most carcinogenic and most abundantly produced of the aflatoxins. However, fluorescent peaks corresponding to aflatoxin B_2, G_1, and G_2 were visualized during development of this methodology (Figure S1) suggesting it has the potential to quantify all aflatoxin metabolites produced by *A. flavus*, but development of the expanded method will require additional validation.

REFERENCES

1. Horn, B.W. Ecology and population biology of aflatoxigenic fungi in soil. In *Aflatoxin and Food Safety*, Abbas, H.K., Ed. CRC Press: Boca Raton, 2005; Vol. 149, pp. 95-116.

2. Horn, B.W. Biodiversity of *Aspergillus* section *Flavi* in the United States: a review. *Food Addit Contam* **2007**, *24*, 1088–1101.

3. Carbone, I.; Ramirez-Prado, J.H.; Jakobek, J.L.; Horn, B.W. Gene duplication, modularity and adaptation in the evolution of the aflatoxin gene cluster. *BMC Evol. Biol.* **2007**, *7*, 111, doi:10.1186/1471-2148-7-111.

4. Wicklow, D.T.; Shotwell, O.L. Intrafungal distribution of aflatoxins among conidia and sclerotia of *Aspergillus flavus* and *Aspergillus parasiticus*. *Can J Microbiol* **1983**, *29*, 1-5.
5. *Action Levels for Aflatoxins in Animal Feeds;* Compliance Policy Guides 683.100, U.S. Food & Drug Administration, 1994.

6. *Adulteration with Aflatoxin;* Compliance Policy Guides 555.400, U.S. Food & Drug Administration, 2005.

7. Khalil, M.M.; Gomaa, A.M.; Sebaei, A.S. Reliable HPLC determination of aflatoxin M1 in eggs. *Journal of analytical methods in chemistry* **2013**, *2013*.

8. Wacoo, A.P.; Wendiro, D.; Vuzi, P.C.; Hawumba, J.F. Methods for detection of aflatoxins in agricultural food crops. *Journal of Applied Chemistry* **2014**, *2014*.

9. Moore, G.G.; Singh, R.; Horn, B.W.; Carbone, I. Recombination and lineage-specific gene loss in the aflatoxin gene cluster of *Aspergillus flavus*. *Mol. Ecol.* **2009**, *18*, 4870-4887.

10. Horn, B.W.; Dorner, J.W. Regional differences in production of aflatoxin B_1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Appl Environ Microbiol* **1999**, *65*, 1444-1449.

11. Horn, B.W. Ecology and population biology of aflatoxigenic fungi in soil. *Journal of Toxicology: Toxin Reviews* **2003**, *22*, 351-379.

12. Carbone, I.; Jakobek, J.L.; Ramirez-Prado, J.H.; Horn, B.W. Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. *Mol. Ecol.* **2007**, *16*, 4401–4417.

13. Drott, M.T.; Lazzaro, B.P.; Brown, D.L.; Carbone, I.; Milgroom, M.G. Balancing selection for aflatoxin in Aspergillus flavus is maintained through interference competition with, and fungivory by insects. *Proc Biol Sci* **2017**, *284*, doi:10.1098/rspb.2017.2408.

14. Faustinelli, P.C.; Palencia, E.R.; Sobolev, V.S.; Horn, B.W.; Sheppard, H.T.; Lamb, M.C.; Wang, X.M.; Scheffler, B.E.; Martinez Castillo, J.; Arias, R.S. Study of the genetic diversity of the aflatoxin biosynthesis cluster in Aspergillus section Flavi using insertion/deletion markers in peanut seeds from Georgia, USA. *Mycologia* **2017**, *109*, 200-209, doi:10.1080/00275514.2017.1307095.

15. Olarte, R.A.; Horn, B.W.; Dorner, J.W.; Monacell, J.T.; Singh, R.; Stone, E.A.; Carbone, I. Effect of sexual recombination on population diversity in aflatoxin production by Aspergillus flavus and evidence for cryptic heterokaryosis. *Mol. Ecol.* **2012**, *21*, 1453-1476, doi:10.1111/j.1365-294X.2011.05398.x.

16. Sobolev, V.S.; Dorner, J.W. Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. *Journal of AOAC International* **2002**, *85*, 642-645.

17. Horn, B.W.; Greene, R.L.; Sobolev, V.S.; Dorner, J.W.; Powell, J.H.; Layton, R.C. Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus, A. parasiticus,* and *A. tamarii*. *Mycologia* **1996**, *88*, 574–587.

18. Mehrzad, J.; Malvandi, A.M.; Alipour, M.; Hosseinkhani, S. Environmentally relevant level of aflatoxin B1 elicits toxic pro-inflammatory response in murine CNS-derived cells. *Toxicology letters* **2017**, *279*, 96-106.

19. Koehler, P.; Hanlin, R.; Beraha, L. Production of aflatoxins B1 and G1 by Aspergillus flavus and Aspergillus parasiticus isolated from market pecans. *Applied microbiology* **1975**, *30*, 581-583.

20. Huang, J.; Elmashni, D. Analysis of aflatoxins using fluorescence detection. In *In "Thermo Scientific Application Note 381"*. 2007.

21. Molo, M.S. Population genetics of biological control in *Aspergillus flavus*. NC State University, 2018.

22. Olarte, R.A.; Worthington, C.J.; Horn, B.W.; Moore, G.G.; Singh, R.; Monacell, J.T.; Dorner, J.W.; Stone, E.A.; Xie, D.Y.; Carbone, I. Enhanced diversity and aflatoxigenicity in interspecific hybrids of Aspergillus flavus and Aspergillus parasiticus. *Mol. Ecol.* **2015**, *24*, 1889-1909, doi:10.1111/mec.13153.

23. Fountain, J.; Scully, B.; Ni, X.; Kemerait, R.; Lee, D.; Chen, Z.-Y.; Guo, B. Environmental influences on maize-Aspergillus flavus interactions and aflatoxin production. *Frontiers in microbiology* **2014**, *5*, 40.

24. Geiser, D.M.; Dorner, J.W.; Horn, B.W.; Taylor, J.W. The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fungal Genet Biol* **2000**, *31*, 169-179.

25. Moore, G.G.; Olarte, R.A.; Horn, B.W.; Elliott, J.L.; Singh, R.; O'Neal, C.J.; Carbone, I. Global population structure and adaptive evolution of aflatoxin-producing fungi. *Ecol Evol* **2017**, *7*, 9179-9191, doi:10.1002/ece3.3464.

Table 3.1 Aflatoxin concentrations and recovery in experimental and spiked conditions.

Spiked Solution	Strain	Spiked Aflatoxin B_1 Conc. (μg/mL)	Recovered Aflatoxin B_1 Conc.[1] (μg/mL)	% Recovery	Avg. % Recovery by Media	Std. Dev % Recovery by Media
YES Media	IC201	1	0.7	70.00	73.27	2.70
YES Media	IC201	6	4.28	71.33		
YES Media	IC201	20	15.02	75.10		
YES Media	IC201	50	38.32	76.64		
YES Media	IC1179	1	0.74	74.00	76.92	9.49
YES Media	IC1179	6	4.1	68.33		
YES Media	IC1179	20	14.48	72.40		
YES Media	IC1179	50	46.48	92.96		
YES Media	Not Inoculated	1	1.02	102.00	84.53	12.89
YES Media	Not Inoculated	6	4.12	68.67		
YES Media	Not Inoculated	20	18.2	91.00		
YES Media	Not Inoculated	50	38.22	76.44		
Chloroform	Not Inoculated	1	0.72	72.00	76.00	5.40
Chloroform	Not Inoculated	6	4.28	71.33		
Chloroform	Not Inoculated	20	15.16	75.80		
Chloroform	Not Inoculated	50	42.44	84.88		

[1]The aflatoxin concentrations obtained from HPLC correspond to the 1 mL of methanol in which dried aflatoxin samples are resuspended. This only contains the amount of aflatoxins within 0.5 mL of the chloroform, which is half of the initial 1.0 mL of chloroform introduced to each sample. To represent the aflatoxin concentration within 1.0 mL of culture, the concentrations reported were obtained by doubling each concentration initially conveyed by HPLC results to factor in the previous halving of that concentration.

Table 3.2 Aflatoxin B_1 concentrations from liquid culture of known aflatoxigenic strains.

NRRL Number	IC Number	B_1 Conc.[1] (µg/mL)	Avg. B_1 Conc. (µg/mL)	Relative Standard Deviation (%)
		0.4		
20025	IC218	0.2	0.29	34.32
		0.28		
		0.16		
20027	IC225	0.08	0.17	58.08
		0.28		
		1.06		
29459	IC229	1.1	1.11	4.55
		1.16		
		29.58		
29507	IC278	27.36	28.76	4.25
		29.36		
		0.36		
29537	IC308	0.2	0.25	36.46
		0.2		

[1]For an explanation of aflatoxin concentrations see footnote of Table 3.1.

Table 3.S1 Aflatoxin B_1 concentrations from liquid culture of *A. flavus* population sample isolates by genetic lineage.

IC Number	Lineage	B_1 Conc.[1] (µg/mL)	Average AF B_1 Conc. (µg/mL)	Relative Standard Deviation (%)
IC6510	IC	1.70 0.16 ND	0.93	117.60
IC6511	IC	33.74 44.76 38.88	39.12	14.09
IC14609	IC	1.57 0.97 0.73	1.09	39.70
IC14611	IC	0.13 ND 0.14	0.13	5.42
IC14613	IC	0.59 0.70 0.68	0.65	8.88
IC14645	IC	ND ND ND	NA	NA
IC14651	IC	ND ND ND	NA	NA
IC14674	IC	0.15 0.05 0.23	0.14	63.35
IC14677	IC	0.09 0.03 ND	0.06	64.07
IC14680	IC	1.46 0.60 2.28	1.45	58.00
IC14683	IC	1.37 2.47 4.60	2.82	58.26
IC14687	IC	0.04 0.14 0.08	0.08	56.47
IC14691	IC	32.73 15.10 25.18	24.34	36.33

Table 3.S1 (continued)

IC14693	IC	0.25 0.58 0.48	0.44	38.80
IC14722	IC	28.31 30.06 22.97	27.11	13.62
IC14756	IC	49.41 24.80 25.48	33.23	42.20
IC14757	IC	10.94 14.31 10.96	12.07	16.10
IC14770	IC	24.78 15.80 21.89	20.82	22.01
IC6512	IB	0.72 2.72 2.54	1.99	55.45
IC6542	IB	0.03 0.03 0.03	0.03	4.33
IC14618	IB	0.50 0.11 0.09	0.23	99.73
IC14631	IB	0.00 0.01 0.08	0.03	140.70
IC14638	IB	0.02 0.01 0.03	0.02	43.71
IC14649	IB	0.02 0.02 0.02	0.02	2.67
IC14650	IB	0.06 0.22 0.03	0.10	98.05
IC14658	IB	0.02 0.02 0.03	0.02	5.88
IC14661	IB	ND ND ND	NA	NA

Table 3.S1 (continued)

IC14664	**IB**	**0.02** 0.02 0.03	**0.02**	**6.89**
IC14676	IB	ND ND ND	NA	NA
IC14684	IB	ND ND ND	NA	NA
IC14688	IB	ND ND ND	NA	NA
IC14697	IB	0.00 0.00 0.06	0.02	150.01
IC14707	IB	0.49 0.05 0.09	0.21	115.17
IC14713	IB	ND ND ND	NA	NA
IC14721	IB	ND ND ND	NA	NA
IC14725	IB	0.09 0.06 0.04	0.06	40.17
IC14728	IB	0.22 0.24 0.48	0.31	46.39
IC14744	IB	0.23 0.03 0.10	0.12	86.60
IC14745	IB	ND ND ND	NA	NA
IC14753	IB	0.44 0.51 0.57	0.51	12.87
IC14768	IB	ND ND ND	NA	NA

Table 3.S1 (continued)

IC14769	IB	0.13 0.07 0.27	0.16	64.24

ND, not detected; NA not applicable

[1]The aflatoxin concentrations obtained from HPLC correspond to the 1 mL of methanol in which dried aflatoxin samples are resuspended. This only contains the amount of aflatoxins within 0.5 mL of the chloroform, which is half of the initial 1.0 mL of chloroform introduced to each sample. To represent the aflatoxin concentration within 1.0 mL of culture, the concentrations reported were obtained by doubling each concentration initially conveyed by HPLC results to factor in the previous halving of that concentration.

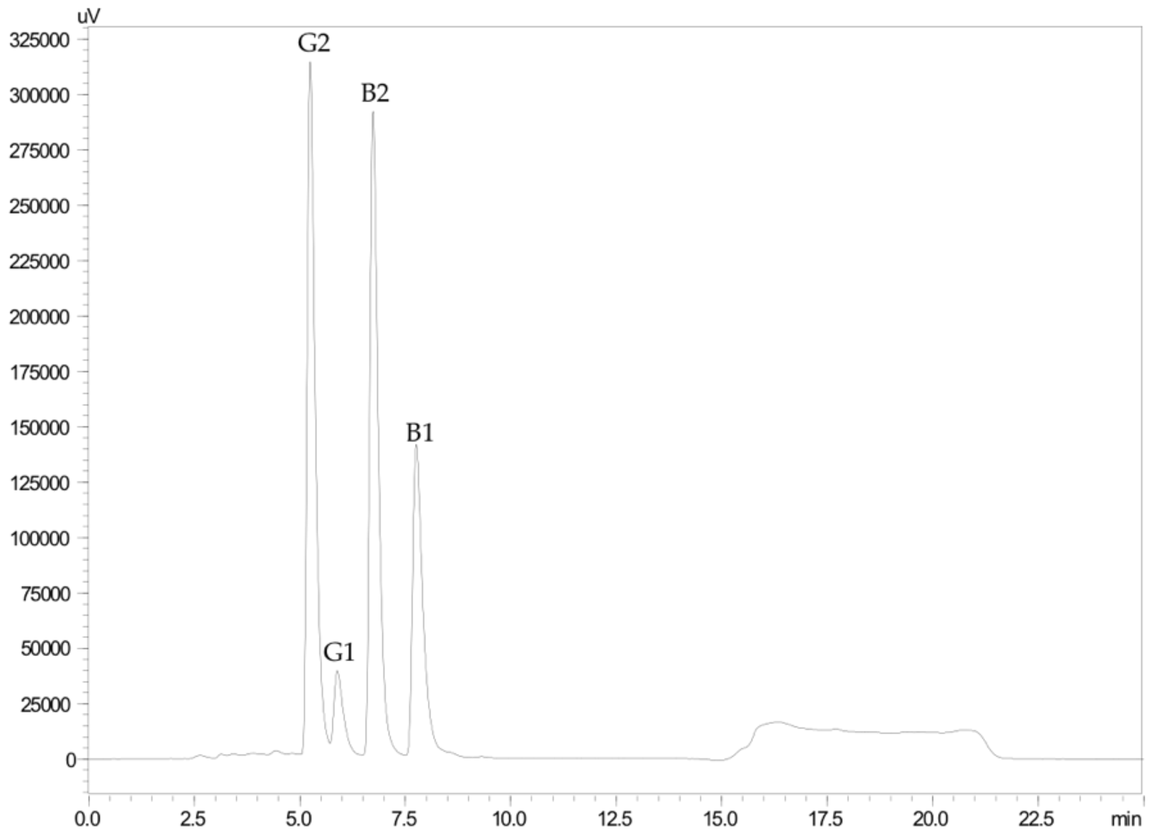


Figure 3.S1 Aflatoxin typical standard chromatogram with aflatoxin B_1 at 0.5 µg/mL, B_2 at 0.25 µg/mL, G_1 at 0.5 µg/mL, and G_2 at 0.05 µg/mL.

CHAPTER 4

Genetic Map and Heritability of *Aspergillus flavus*

Submitted to Fungal Genetics and Biology

Richard M Gell[1*], Bruce W. Horn[2], and Ignazio Carbone[1]

[1]Center for Integrated Fungal Research, Program of Genetics, Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA.

[2]National Peanut Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Dawson, Georgia, United States of America.

*Corresponding author (rmgell@ncsu.edu)

CONTRIBUTION SUMMARY

For the experiments in this paper, Dr. Ignazio Carbone, Dr. Eric Stone, and I conducted the initial concept and approach for identifying the genetic maps. I extracted DNA and processed the samples for sequencing with the assistance of Vicki Cornish. I designed and performed the initial filtration and variant identification on Mobyle SNAP Workbench, processed the samples through the adapted R/qtl pipeline, wrote and edited the manuscript with Dr. Ignazio Carbone.

ABSTRACT

The carcinogenic mycotoxin aflatoxin is a constant threat and economic burden to corn and oil seed crops grown within the United States and globally. Aflatoxin is produced by fungi species in *Aspergillus* section *Flavi*, primarily *Aspergillus flavus*. Though previously thought of as only asexual, *A. flavus* has recently been found to undergo sexual reproduction both in laboratory crosses and in the field. To gain a greater understanding of the effects of the sexual cycle, we constructed genetic maps of *A. flavus* based on three mapping populations each composed of the parental strains and approximately 70 F1 progeny. Genome wide data using double digest Restriction Associated DNA sequencing identified 496, 811, and 576 significant polymorphisms differentiating parents across eight linkage groups to serve as markers. Average spacing between marker loci was 3.1, 2.1, and 3.5 map units and overall map length was 1504.4, 1669.2, and 2001.3 cM. Recombination events were non-randomly distributed across chromosomes with an average rate of recombination of about 46.81 cM per Mbp. We show anisogamous inheritance of mitochondrial loci from the sclerotial strain in crosses, whereas nuclear loci show a 1:1 segregation ratio from both parents. The linkage map will be useful in QTL analyses to identify traits that both increase sexual fertility in *A. flavus* and modulate aflatoxin production, which have significant implications for sustainable reduction of aflatoxin contamination using biological control agents.

INTRODUCTION

Aflatoxin is one of the most potent mycotoxins that is acutely toxic to humans and animals (Williams *et al.* 2004). Aflatoxins are produced by *Aspergillus flavus* and several other species in *Aspergillus* section *Flavi* during contamination of many important crops worldwide leading to large economic losses. *A. flavus* can be further subdivided into two distinct evolutionary lineages, designated as lineage IB and IC (Moore *et al.* 2009). Lineage IB strains are predominantly clonal, non-aflatoxigenic or producing very low aflatoxin levels; lineage IC strains vary widely in their ability to produce aflatoxins, ranging from those that are non-aflatoxigenic to those that are potent producers of aflatoxins (Carbone *et al.* 2007; Drott *et al.* 2017; Horn 2003; Horn & Dorner 1999). Current biological control methods reduce toxicity in fields by releasing a high-density application of a single *A. flavus* non-aflatoxin producing strain. This results in a transient and artificial increase in clonality in local populations and significant reductions in aflatoxin levels (Dorner 2004). Less certain is the impact of biocontrol on genetic exchange and recombination in fields and the possibility of leveraging sexual reproduction in biocontrol strategies.

A. flavus is capable of heterothallic sexual reproduction under laboratory conditions and in the field with variable rates of fertility utilizing the interaction between mating type loci *MAT1-1* and *MAT 1-2* (Horn *et al.* 2016; Horn *et al.* 2009; Ramirez-Prado *et al.* 2008). During the mating process, the sclerotium, a survival structure, of one strain acts as the female parent providing both the mitochondria and a matrix for the ascocarps in which the progeny grow, while a spore or propagule from a second compatible strain fertilizes as the male (Horn *et al.* 2016). The fertility of mating pairs is highly variable and strongly influenced by the directionality of the cross, but little is known about the genetic makeup of first generation progeny and how

inheritance and recombination is regulated. In crosses that exhibit the strong bias in fecundity, there is a question about if the nuclear genome segregates independently or if that bias is reflected in the genetics of the viable offspring. This could impact male and female fertility (Dyer & O'gorman 2012), sexual compatibility (Leslie & Raju 1985; Raju 1992) or inheritance of strictly biparental traits.

Previous studies into genetic heritability within *Aspergillus flavus* have focused primarily on regions within the aflatoxin gene cluster which supported the occurrence of recombination along these regions as hotspots (Olarte *et al.* 2012). Multi-locus sequencing data for regions outside the gene cluster in many offspring (Horn *et al.* 2016; Olarte *et al.* 2012) suggest independent assortment of chromosomes due to absence of association between markers on different chromosomes. The position of recombination events along chromosomes was further examined with nine parent-offspring trios using array comparative genome hybridization and suggested the commonness of crossover events along each chromosome. However, this methodology was short on resolution outside of the aflatoxin gene cluster, parentage was often too similar and therefore difficult to distinguish, and too costly for proper sample sizes of a large numbers of progeny from a single cross (Olarte *et al.* 2012).

In order to gain greater understanding of the sexual cycle in *A. flavus,* explore the influence of directionality in the offspring and determine what genetic heritability appears as genome wide for the first time, the full set of first generation progeny from a previous study was examined (Horn *et al.* 2016). This provides a larger sample size for testing the hypothesis of uniparental mitochondrial inheritance from the sclerotial strain. Through the use of reduced representative genome sequencing, polymorphisms from across the genome are used as markers to determine parental origin for each. This data was used to construct genetic maps of *A. flavus*

and compared with the physical map to determine recombination rates across the genome. These can also be useful in examining the association between parental role and genomic inheritance and serve as a resource for more broadly identifying genes that are important in aflatoxin production, sexual fertility and other traits of interest.

MATERIALS AND METHODS

Fungal Strains

We examined three crosses: IC244 (=NRRL 29473) × IC1179 (=NRRL 18543), IC308 (=NRRL 29537) × IC307 (=NRRL 29536), and IC278 (=NRRL 29507) × IC201 (=NRRL 21882) that were previously shown to exhibit high fertility in one direction, but low fertility when male and female parents are reversed (Horn *et al.* 2016). Briefly, *A. flavus* sclerotia from single strains of one mating type were incubated on sterile soil to which a solution containing conidia, an asexual spore, from a strain of the opposite mating type had been added. The cross was also conducted in the reciprocal direction as described previously (Horn et al. 2016). We developed three mapping populations comprising F1 progeny from crosses of parental lines that differed in female fertility and aflatoxin production, including the non-aflatoxigenic *A. flavus* biocontrol strains IC201 (=Afla-Guard; NRRL 21882) and IC1179 (=AF36; NRRL 18543). To explore the impact of genomic similarity, two crosses (IC244 × IC1179 and IC308 × IC307) had both parents from the same lineage within *A. flavus*, IC, while in a third cross (IC278 × IC201) each parent came from different lineages with IC278 from lineage IC and IC201 from lineage IB.

Mitochondrial Variants

Following the approach of Horn *et al.* 2016, AF-MIT-1 (F: TGAAGCAACTGGATTATTCGCA, R: AAACCACATTCAAAAGCGCT), AF-MIT-3 (F: AGCAGAGGGTTCTGCGTTT, R: GCAGATCAACCTGCTAATAATATTCC) and AF-MIT-4 (F: GCTAAAGTTATAGGAGGTGAAGT, R: GCAACCTTTAGCTTCAATAAACCC) were used to amplify known mitochondrial polymorphisms in IC308 × IC307, IC278 × IC201, and IC244 × IC1179, respectively. Sequencing was performed by the North Carolina State University Genomic Sciences Laboratory, alignments were constructed with SEQUENCHER version 4.7

(Gene Codes Corporation, Ann Arbor, MI), and haplotypes were called using the same methodologies as previously described (Horn *et al.* 2016).

DNA Sequencing

Total genomic DNA was isolated for 207 strains using MoBio's UltraClean® Microbial DNA Kit (Catalog# 12224-250). DNA was subjected to double digest Restriction site Associated DNA Sequencing (ddRADSeq), which is a genotyping-by-sequencing approach (Peterson *et al.* 2012) that uses restriction enzyme digestion and size fractionation to reduce the *A. flavus* genome size of each strain from about 37 Mb to approximately 2 Mb. Briefly, DNA was digested with two restriction enzymes (MluC1 and Msp1) and adapters were then ligated to the end of the DNA fragments. The adapters include one of 48 barcodes, which allow tracking of individual strains. After a sizing step to capture DNA fragments in the 300-400 bp range, the fragments are subjected to PCR amplification to introduce Illumina adaptors and then sequenced using 125 bp paired end reads on the NextSeq Illumina sequencer platform.

Genotype Calling and Mapping

Genotyping-by-sequencing data was analyzed using workflows implemented in the Mobyle SNAP Workbench (Monacell & Carbone 2014). Briefly, the process_radtags script from the Stacks package (http://catchenlab.life.illinois.edu/stacks/) was used to demultiplex barcodes for each Illumina NextSeq sublibrary (Catchen *et al.* 2013). Trimmomatic (Bolger *et al.* 2014) was used to quality trim and crop read pairs. Filtered read pairs were aligned to the *A. oryzae* RIB40 reference genome using the MEM algorithm in BWA (Li & Durbin 2009); *A. oryzae* RIB40 was selected as the reference genome due to the high quality of the genome, robust annotation, and the close genetic relationship between *A. oryzae and A. flavus* (Machida *et al.* 2005). Sequence alignment files for parental strains and their associated progeny generated

from BWA were assembled into cohorts and genotyped using the HaplotypeCaller variant discovery pipeline in GATK v3.5-2 (McKenna *et al.* 2010). GATK variant calling is designed to maximize sensitivity, so there could be many false positives. Subsequent filtering of variants using a minor allele frequency of 0.1 and a max missing of 0.9 was performed using VCFtools (https://vcftools.github.io/index.html); this was important to eliminate false positives and negatives and was performed according to GATK Best Practices recommendations (DePristo *et al.* 2011; Van der Auwera *et al.* 2013). Variant call sets generated for each cross were visualized in JBrowse (Skinner *et al.* 2009).

The filtered variant call files produced were indexed using the bgzip and indexTabix commands in Rsamtools and converted using the vcf2raw command in onemap (Margarido *et al.* 2007; Morgan 2011). Chromosome association, marker names, strain names, and parental allele calls were extracted from the raw file and formatted to csv_rot for importing into the R/qtl package (Broman *et al.* 2003). Duplicate and low yield markers and low coverage progeny strains were identified and excluded from the analysis. The SNP markers for each progeny strain were compared with the markers for each other strain from that cross to calculate the genome wide similarity in shared SNP calls between each pair of sibling progeny strains. For each pair that shared greater than 95% of SNP calls, one strain was randomly removed from the analysis as a duplicate individual.

Recombination Fractions and Determining Linkage

The function est.rf from the R/qtl package initially determined the recombination fractions for each pair of markers which were ordered based on location determined by their physical location on the RIB40 genome (Broman *et al.* 2003; Machida *et al.* 2005). These were visualized using the plotRF function where inconsistencies in the recombination frequency can

identify any regions where adjustment of marker order to reflect frequency of inheritance from the same parental strain may be required. The est.map function using the haldane mapping approach was used to determine the initial genetic distances between adjacent markers as ordered by the RIB40 genome; errors in the positioning of markers on chromosomes may be identified at this step if chromosomes show genetic distances substantially larger than chromosomes of similar physical size.

For problematic chromosomes, if markers show no linkage to an adjacent region of markers but potential linkage to markers on another chromosome these markers can be distributed to new chromosomes. For these crosses the markers from chromosomes 2 and 6 in each dataset were subsetted out of the whole genome data and processed with the formLinkageGroups function using values max.rf of 0.25 and min.LoD of 6.0 for IC244 × IC1179 and IC308 × IC307, but a max.rf of 0.30 and min.LoD of 3.0 for IC278 × IC1179 to determine how the markers from these chromosomes were linked and their relative genetic map positions. Based on this new grouping the markers in the full dataset were adjusted to their new genetic map positions.

Recombination Rate

The initial physical map of marker positions from the RIB40 genome was provided in the csv_rot. By moving markers to their new relative position for chromosomes 2 and 6 within the csv_rot the final physical map of marker positions was produced.

For markers from RIB40's chromosome 2 determined to be on chromosome 6, the physical position was determined by their physical distance from the translocation position plus the physical location of the translocation point. The closest marker to the translocation point from RIB40's chromosome 2 for each cross was treated as having a distance from the

translocation point of 0 Mb, and the distance to the translocation point for the other markers of that cross were determined by subtracting their initial physical location from the initial physical location of the closest marker. The physical location of the translocation point was treated as 3.00 Mb on chromosome 6, due to this location being a viable option for all crosses but could be between 2.97Mb and 3.04Mb which was the window between RIB40 physical locations for markers that were part of the translocation and markers which supported staying on chromosome 6 of the smallest size from the IC308 × IC307 cross.

The portion of RIB40's chromosome 6 being repositioned onto chromosome 2 was larger than the piece which was removed so all physical positions of markers on chromosome 2 were increased 0.2 Mb to allow for space. For markers from chromosome 6 to chromosome 2, physical location was determined by the distance of the marker from the translocation position on chromosome 6 subtracted from the expected translocation physical position along chromosome 2.

The same markers and individuals that were removed during the development of the genetic map for being duplicates or containing poor data were removed from the physical map as well. The est.recrate function between each genotype map and physical map was run using sliding window of 500 kb across each chromosome. The r/xoi program's recrate2scanone function was used to plot this variation across the chromosomes (Broman & Kwak 2018).

RESULTS

Mitochondrial Calls

Of the 177 first generation progeny with mitochondrial sequences with sufficient quality to align against their parental reference sequences, 176 had the sequence polymorphisms associated with the parental strain which was introduced as sclerotia to the cross. For IC244 × IC1179, 33 progeny strains had the SNP associated with the IC244 mitochondrial genome from the portion of the cross where IC244 acted as the sclerotial strain. All 24 progeny strains with the variant associated with IC1179 came from the portion of the cross where IC1179 acted as the sclerotial strain. Similar results were observed from the IC278 × IC201 set where all 26 progeny strains with polymorphisms matching IC278 came from crosses where IC278 was the sclerotial strain and all 32 progeny strains with IC201-like mitochondria came from crosses with IC201 as the sclerotial strain. In the IC308 × IC307 cross, all 33 progeny strains with IC308 as the sclerotial parent had IC308-like mitochondria. Additionally, one strain with IC307 as the sclerotial parent, IC5858, also showed the polymorphism associated with IC308 mitochondria. Only 28 of the 29 progeny strains with IC307 as the sclerotial parent gave sequences consistent with IC307-like mitochondria.

Progeny Statistics

For each cross, the number of progeny strains and parentally distinguishable SNPs (i.e., the markers) obtained initially from the aligned and sorted ddRADseq data as well as the final counts of unique viable progeny datasets and parental markers for each mapping cross are shown in Table 1. The largest loss of markers was due to the high presence of marker pairs where each progeny strain had the same parental call at both positions; 534, 496, and 565 markers were dropped in IC244 × IC1179, IC308 × IC307, and IC278 × IC201 datasets, respectively. Certain

progeny strains were removed due to low quality data as evidenced by appearing to contain more than 100 crossover events (one strain from IC244 × IC1179, three strains from IC308 × IC307, and three strains from IC278 × IC201). One of each pair of any duplicate progeny strains was removed as identified by the strains sharing greater than 95% of marker calls (two strains from IC244 × IC1179, five strains from IC308 × IC307, and five strains from IC278 × IC201 strains). Any strains that were discovered to be identical at all markers with a parental strain were dropped from the study (one strain from IC244 × IC1179).

Frequency of marker calls associated with the *MAT1-1* parental strain and the *MAT1-2* parental strain were calculated by R/qtl and approached equilibrium for overall markers within each cross with IC244 × IC1179 at 51.8% to 48.2%, IC308 × IC307 at 51.0% to 49.0%, and IC278 × IC201 at 48.6% to 51.4%. For each progeny strain the proportion of markers associated with each parental strain was less consistent. While the majority of progeny strains examined had between 40% and 60% of markers associated with each parent, many contained marker proportions that were more extreme with examples of progeny strains containing over 80% of markers associated with a parental strain present in multiple crosses (Figure 1).

The initial alignment of markers allowed for a calculation of the genetic length of each chromosome based on the order the markers occurred within the published *A. oryzae* RIB40 reference genome (Machida *et al.* 2005). The genetic distances for chromosomes 2 and 6 based on the RIB40 alignments, appeared an order of magnitude larger than the other six chromosomes in all three crosses (Table 2). Further examination of this initial dataset showed that the maximum spacing between markers reached the cap for r/qtl at 1001.5 cM on both chromosomes in all three crosses. All alignments to RIB40 were deposited in the sequence read archive at Genbank: PRJNA534054.

Recombination Fractions and Determining Linkage

Recombination fraction analysis showed that the observed recombination fractions between each marker matched the expected amount except for two regions on chromosomes 2 and 6 (Figure 2A-C; Supplementary Figure S1). Markers for these chromosomes were grouped and sorted into new chromosomes based on linkage association and then ordered based on a simple model in which an inverted reciprocal translocation between the two chromosomes occurred. The data supports a translocation occurring in the same interval on both chromosomes for all three crosses with marker positions based on the published *A. oryzae* RIB40 genome (Figure 2D-F). The translocation occurred between markers on chromosome 2 at 0.81 Mb and 1.06 Mb for IC244 × IC1179 cross, at 0.98 Mb and 1.02 Mb for the IC308 × IC307 cross, and at 0.92 Mb and 1.02 Mb for the IC278 × IC201 cross. Markers on chromosome 6 showed the rearrangement occurring between 2.82 Mb to 3.14 Mb for IC244 × IC1179 cross, 2.97 Mb to 3.04 Mb for the IC308 × IC307 cross, and 2.84 Mb to 3.03 Mb for the IC278 × IC201 cross.

The organization of markers implementing this reciprocal inverted translocation between chromosomes 2 and 6 is usually a model that minimizes the total number of obligate crossovers required for each chromosome. The greatest difference was observed on chromosome 2 of the IC308 × IC307 cross where it required 183 crossovers to have occurred in all progeny strains along the chromosome but through additional rearrangement of chromosome 2 markers could require only 179 crossover events to have occurred among all progeny strains. Due to the inconsistency across the crosses of potential minor rearrangements of chromosome 2 and 6 markers as well as the absence of further significant deviations in the recombination fraction results, the model using only the reciprocal translocation was used to determine the final genetic maps with other markers continuing to be ordered based on their initial genome alignment.

Because the majority of markers and genetic length continued to correspond to specific chromosomes in the RIB40 genome, no renaming or reorganization of the chromosomes due to new expected lengths or contents was conducted to maintain synteny. The chromosome containing markers from the 3' region of chromosome 2 to the translocation point near 1 Mb, based on the RIB40 alignment, will continue to be called chromosome 2 despite also containing the markers from the translocation point around 3 Mb position of Chromosome 6 to its 3' most region. Similarly, the chromosome containing markers from the 5' most position of chromosome 6 to the translocation position on chromosome 6 and the translocation position on chromosome 2 to its 5' most marker from the RIB40 alignment will continue to be referred to as chromosome 6 in this new chromosome organization (Figure 3, Supplementary Figure S2).

Genetic Maps

Maps representing the relative position of all markers along chromosomes in *A. flavus* for each cross based on their degree of genetic linkage are shown in Figure 4. These maps, developed with the inverted translocation, show greatly reduced lengths corresponding to the reorganization of chromosomes 2 and 6 markers (Table 3). These reorganized chromosomes are well within the ranges of lengths for the other chromosomes of each cross presented in Table 2. Regions around the inverted translocation still contain the largest genetic distance between markers for that chromosome in most crosses; such as the region between marker Chr6_3140858, the marker nearest the translocation from chromosome 6 to chromosome 2, and Chr6_3577894 corresponding to a largest gap of 49.1 cM on chromosome 2 of the IC244 × IC1179 cross. This greatest distance between markers for the reorganized chromosomes was within the range of greatest distances between markers for its associated cross where they had maxed out r/qtl's est.map function at 1001.5 cM previously.

Recombination Rate

An overall estimate of recombination rate for each cross was calculated by taking the total genetic length in centimorgans and dividing it by the total physical distance covered by markers in that cross. For the three crosses, the total genetic distance was 1504.4 cM for IC244 × IC1179, 1669.2 cM for IC308 × IC307, and 2001.3 cM for IC278 × IC201. The total physical distances utilized were 37.1 Mb, 37.3 Mb, and 36.3 Mb resulting in recombination rate estimates of 40.55, 44.75, and 55.13 cM per Mb, respectively. Though these numbers allow for a quick point of comparison between the crosses, a better understanding of recombination rate can be obtained through examination of the variation in recombination rate through sliding windows around points distributed across each chromosome (Figure 5).

DISCUSSION

This is the first genetic map of *A. flavus.* This work shows that sexual reproduction is very efficient in generating new *A. flavus* genotypes in a single generation. On average, a progeny strain after a single mating event is expected to contain between 15 and 20 recombination events distributed across the genome. Inheritance for each progeny strain is on average 50% from each parental strain and each chromosome undergoes a little more than two recombination events per generation in each progeny.

In nature, *A. flavus* strains show evidence of admixture even though populations are strongly structured by evolutionary lineage, either IB or IC (Molo 2018). Although the interlineage cross between the IC strain IC278 and the IB strain IC201 (=Afla-Guard; NRRL 21882) showed a larger total genetic length at 2001.3 cM and therefore a proportionally higher recombination rate at 55.13 cM per Mb, there were no other noticeable unique qualities when comparing it to either of the two within lineage crosses between IC244 and IC1179 (=AF36; NRRL 18543) at 1504.4 cM and 40.55 cM/Mb or IC308 and IC307 at 1669.2 cM and 44.75 cM/Mb. The observed random inheritance of markers in progeny strains from the interlineage cross (Figure 1) suggests variables other than nonrandom recombination, such as incompatible sexual cycles or limited co-localization in fields, are sufficient for maintaining *A. flavus* lineage structure; however, once those forces are surpassed meiosis is capable of recombining the two lineages.

The recombination rates identified for *A. flavus* are consistent with estimates reported across the fungal kingdom with an average of 48.68 cM/Mb across fifteen fungal species (Stapley *et al.* 2017). This estimate includes the related *Aspergillus nidulans* which also has eight chromosomes, and a genetic length of 3624.7 cM and physical length of 30.2 Mb (Christians *et*

al. 2011) and the more distantly related *Fusarium verticillioides* with 12 chromosomes and a genetic length of 2188 cM and physical length of 41.8 Mb (Jurgenson *et al.* 2002). The variation in genetic lengths between crosses is also within the range of what has been previously identified within Ascomycota from two crosses utilizing different strains of *Zymoseptoria tritici* reporting genetic lengths of 2723.6 cM and 2158.9 cM (Croll *et al.* 2015).

Recombination rates were not even across chromosomes with regions of elevated recombination corresponding in many cases to subtelomeric segments, whereas recombination was less frequent in many regions near the center of chromosomes, likely associated with their centromeres which is consistent with what is regularly observed across fungi (Barton *et al.* 2008; Croll *et al.* 2015; Stajich *et al.* 2010). For example, the highest recombination rate in both intralineage crosses was observed around the distal right arm of chromosome 3 where the aflatoxin gene cluster is located. This is consistent with previous observations from array comparative genome hybridization data showing frequent recombination events within the gene cluster from population genetic data (Olarte *et al.* 2012).

These genetic maps provide unique insight into the genomic organization of *A. flavus* and how it differs from the published genome of *A. oryzae* RIB40 (Machida *et al.* 2005). The inverted reciprocal translocation between chromosomes 2 and 6 appears to be present in all six parental strains, incorporating both lineages IB and IC, due to its clear presence in all crosses from the detection of crossover events among the translocated regions of the chromosomes. When the sixteen largest scaffolds from the *A. flavus* NRRL 3357 genome, each expected to associate with one arm of the eight chromosomes, were compared with the RIB40 genome, a translocation event in the evolutionary divergence between those genomes was identified between chromosomes 2 and 6 (Payne *et al.* 2006). Genomes for three non-aflatoxin producing

A. flavus strains, including IC201 (=Afla-Guard; NRRL 21882), have been aligned to the genome of NRRL 3357 and no discrepancy in genome organization was present in the translocation regions (Pennerman *et al.* 2018). This inverted reciprocal translocation event was also discovered in *A. oryzae* BCC7051 via comparative genomic analysis with the RIB40 genome (Thammarongtham *et al.* 2018). The evidence for this genomic rearrangement was discovered through multiple methodologies across both lineages of *A. flavus* and within *A. oryzae*. This suggests that the chromosome organization consistent with the linkage maps presented in Figure 3 is the ancestral state which gave rise to contemporary chromosomal structure of *A. oryzae* and *A. flavus*. The ancestor of which that gave rise to RIB40 underwent an inverted reciprocal translocation between chromosomes 2 and 6, potentially during the process of domestication. This becomes relevant when determining choice of markers in population genetics studies of these species, understanding the presence of linkage equilibrium between seemingly adjacent regions, as well as when attempting to compare genome synteny within *Aspergillus* section *Flavi* or other related species.

The three crosses reported in this paper provide the first *A. flavus* reference panel to identify loci that are significant in any variable parental phenotype within these populations. While it is clear that the parental source of sclerotia and conidia influence the fecundity of the cross, the impact that genome context and organellar inheritance have on modulating further phenotypes is unknown. Understanding the patterns of inheritance is particularly important for identifying the factors that contribute to polygenic traits. For example, aflatoxin production is highly heritable (Olarte *et al.* 2012; Olarte *et al.* 2015) and all progeny strains that make aflatoxin inherit an intact biosynthetic cluster (Olarte *et al.* 2012) from a parent but it is also dependent on a polygenic mode of inheritance as several unlinked genes that regulate aflatoxin

biosynthesis have been described (Bok & Keller 2004; Du *et al.* 2007; Kale *et al.* 2008; Payne *et al.* 1993). These progeny populations allow a novel method for new loci and genetic backgrounds to be examined for toxin production, ability to function in toxin reduction, sexual fertility or other distinguishing phenotypes that may enhance the efficacy of *A. flavus* biocontrol. Parental and F1 progeny strains are available to be shared with the community to enable mapping genes in parental strains with different phenotypes or closely related species.

REFERENCES

Barton AB, Pekosz MR, Kurvathi RS, Kaback DB (2008) Meiotic recombination at the ends of chromosomes in *Saccharomyces cerevisiae*. *Genetics* **179**, 1221-1235.

Bok JW, Keller NP (2004) LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell* **3**, 527-535.

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114-2120.

Broman KW, Kwak I-Y (2018) *xoi: Tools for Analyzing Crossover Interference. R Package Version 0.67-4.* https://github.com/kbroman/xoi

Broman KW, Wu H, Sen Ś, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. *Bioinformatics* **19**, 889-890.

Carbone I, Jakobek JL, Ramirez-Prado JH, Horn BW (2007) Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. *Molecular Ecology* **16**, 4401–4417.

Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA (2013) Stacks: an analysis tool set for population genomics. *Molecular Ecology* **22**, 3124-3140.

Christians JK, Cheema MS, Vergara IA*, et al.* (2011) Quantitative trait locus (QTL) mapping reveals a role for unstudied genes in *Aspergillus* virulence. *PLoS One* **6**, e19325.

Croll D, Lendenmann MH, Stewart E, McDonald BA (2015) The impact of recombination hotspots on genome evolution of a fungal plant pathogen. *Genetics* **201**, 1213-1228.

DePristo MA, Banks E, Poplin R*, et al.* (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet* **43**, 491-498.

Dorner JW (2004) Biological Control of Aflatoxin Contamination of Crops. *Journal of Toxicology: Toxin Reviews* **23**, 425-450.

Drott MT, Lazzaro BP, Brown DL, Carbone I, Milgroom MG (2017) Balancing selection for aflatoxin in *Aspergillus flavus* is maintained through interference competition with, and fungivory by insects. *Proc Biol Sci* **284**.

Du W, Obrian GR, Payne GA (2007) Function and regulation of *aflJ* in the accumulation of aflatoxin early pathway intermediate in *Aspergillus flavus*. *Food Addit Contam* **24**, 1043-1050.

Dyer PS, O'gorman CM (2012) Sexual development and cryptic sexuality in fungi: insights from *Aspergillus* species. *FEMS microbiology reviews* **36**, 165-192.

Horn BW (2003) Ecology and population biology of aflatoxigenic fungi in soil. *Journal of Toxicology: Toxin Reviews* **22**, 351-379.

Horn BW, Dorner JW (1999) Regional differences in production of aflatoxin B_1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Appl Environ Microbiol* **65**, 1444-1449.

Horn BW, Gell RM, Singh R, Sorensen RB, Carbone I (2016) Sexual reproduction in *Aspergillus flavus* sclerotia: Acquisition of novel alleles from soil populations and uniparental mitochondrial inheritance. *PLoS One* **11**, e0146169.

Horn BW, Moore GG, Carbone I (2009) Sexual reproduction in *Aspergillus flavus*. *Mycologia* **101**, 423-429.

Jurgenson JE, Zeller KA, Leslie JF (2002) Expanded genetic map of *Gibberella moniliformis* (*Fusarium verticillioides*). *Appl. Environ. Microbiol.* **68**, 1972-1979.

Kale SP, Milde L, Trapp MK*, et al.* (2008) Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. *Fungal Genetics and Biology* **45**, 1422-1429.

Leslie JF, Raju NB (1985) Recessive mutations from natural populations of *Neurospora crassa* that are expressed in the sexual diplophase. *Genetics* **111**, 759–777.

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-1760.

Machida M, Asai K, Sano M*, et al.* (2005) Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **438**, 1157-1161.

Margarido GR, Souza AP, Garcia AA (2007) OneMap: software for genetic mapping in outcrossing species. *Hereditas* **144**, 78-79.

McKenna A, Hanna M, Banks E*, et al.* (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome research* **20**, 1297-1303.

Molo MS (2018) *Population genetics of biological control in Aspergillus flavus*, North Carolina State University.

Monacell JT, Carbone I (2014) Mobyle SNAP Workbench: a web-based analysis portal for population genetics and evolutionary genomics. *Bioinformatics* **30**, 1488-1490.

Moore GG, Singh R, Horn BW, Carbone I (2009) Recombination and lineage-specific gene loss in the aflatoxin gene cluster of *Aspergillus flavus*. *Molecular Ecology* **18**, 4870-4887.

Morgan M (2011) *An introduction to Rsamtools.* https://bioconductor.org/packages/release/bioc/vignettes/Rsamtools/inst/doc/Rsamtools-Overview.pdf

Olarte RA, Horn BW, Dorner JW*, et al.* (2012) Effect of sexual recombination on population diversity in aflatoxin production by *Aspergillus flavus* and evidence for cryptic heterokaryosis. *Mol Ecol* **21**, 1453-1476.

Olarte RA, Worthington CJ, Horn BW*, et al.* (2015) Enhanced diversity and aflatoxigenicity in interspecific hybrids of *Aspergillus flavus* and *Aspergillus parasiticus*. *Mol Ecol* **24**, 1889-1909.

Payne G, Nystrom G, Bhatnagar D, Cleveland T, Woloshuk C (1993) Cloning of the *afl-2* gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. *Appl. Environ. Microbiol.* **59**, 156-162.

Payne GA, Nierman WC, Wortman JR*, et al.* (2006) Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. *Medical Mycology* **44**, S9-S11.

Pennerman KK, Gonzalez J, Chenoweth LR*, et al.* (2018) Biocontrol strain *Aspergillus flavus* WRRL 1519 has differences in chromosomal organization and an increased number of transposon-like elements compared to other strains. *Molecular Genetics and Genomics* **293**, 1507-1522.

Peterson BK, Weber JN, Kay EH, Fisher HS, Hoekstra HE (2012) Double Digest RADseq: An Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model Species. *PLoS One* **7**, e37135.

Raju NB (1992) Genetic control of the sexual cycle in *Neurospora*. *Mycological research* **96**, 241-262.

Ramirez-Prado JH, Moore GG, Horn BW, Carbone I (2008) Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genet Biol* **45**, 1292-1299.

Skinner ME, Uzilov AV, Stein LD, Mungall CJ, Holmes IH (2009) JBrowse: a next-generation genome browser. *Genome research* **19**, 1630-1638.

Stajich JE, Wilke SK, Ahrén D*, et al.* (2010) Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea* (*Coprinus cinereus*). *Proceedings of the National Academy of Sciences* **107**, 11889-11894.

Stapley J, Feulner PGD, Johnston SE, Santure AW, Smadja CM (2017) Variation in recombination frequency and distribution across eukaryotes: patterns and processes. *Philosophical Transactions of the Royal Society B: Biological Sciences* **372**, 20160455.

Thammarongtham C, Nookaew I, Vorapreeda T*, et al.* (2018) Genome Characterization of Oleaginous *Aspergillus oryzae* BCC7051: A Potential Fungal-Based Platform for Lipid Production. *Current microbiology* **75**, 57-70.

Van der Auwera GA, Carneiro MO, Hartl C*, et al.* (2013) From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. *Curr Protoc Bioinformatics* **43**, 11.10.11-33.

Williams JH, Phillips TD, Jolly PE*, et al.* (2004) Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am J Clin Nutr* **80**, 1106-1122.

Table 4.1 Initial and filtered progeny individuals and markers

	IC244xIC1179	IC308xIC307	IC278xIC201
Parental Lineages	IC x IC	IC x IC	IC x IB
Initial individuals	71	69	67
Initial Markers	1054	1346	1142
Individuals After Filtering	67	61	59
Markers After Filtering	492	811	576
% MAT1-1 Genotype	51.8	51	48.6

Table 4.2 Initial genetic map lengths in centimorgans for the complete map and each chromosome based on *A. oryzae* RIB40

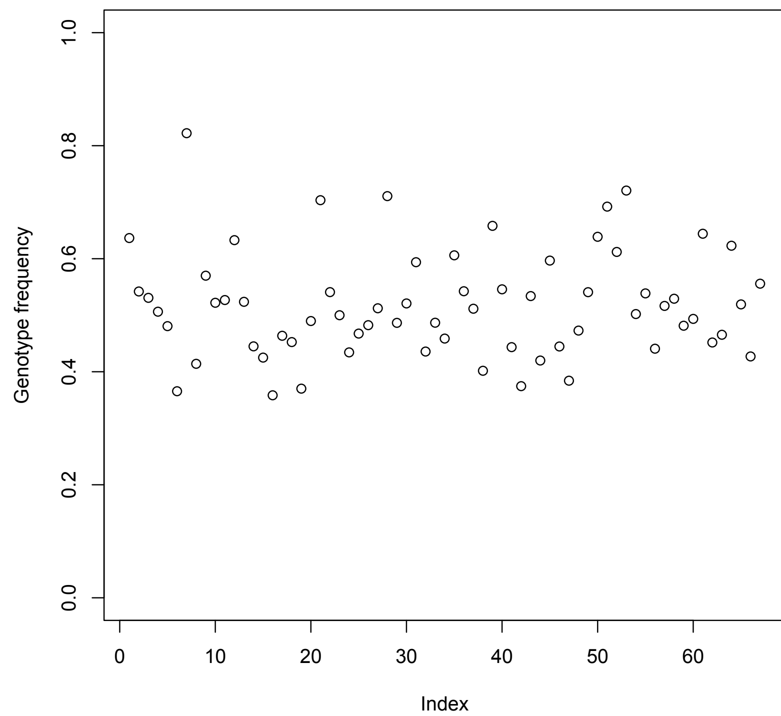
	IC244xIC1179	**IC308xIC307**	**IC278xIC201**
Overall	3480.4	3653.1	3995.7
Chromosome 1	271.3	238.9	346.3
Chromosome 2	1197.8	1196.8	1271.9
Chromosome 3	207.7	233.7	244.7
Chromosome 4	176.9	203.3	247.8
Chromosome 5	175.8	231.9	197.9
Chromosome 6	1193.7	1206.8	1242.5
Chromosome 7	112	141.7	167
Chromosome 8	145.1	200	277.6

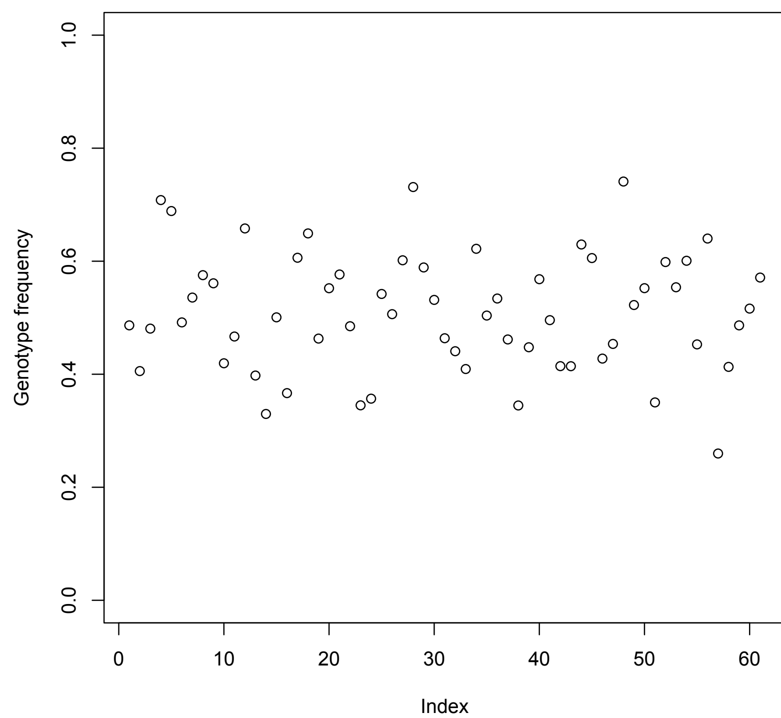
Table 4.3 Comparison of genetic lengths with *A. oryzae* RIB40 marker organization and the new organization for chromosomes 2 and 6

IC244xIC1179	***A. oryzae***	**New**
Overall length (cM)	3480.4	1504.4
Overall avg. spacing (cM)	7.2	3.1
Length of Chr2 (cM)	1197.8	221.1
max distance between markers of Chr2 (cM)	1001.5	49.1
Length of Chr6 (cM)	1193.7	194.2
Max distance between markers of Chr6 (cM)	1001.5	25.1
IC308xIC307	***A. oryzae***	**New**
Overall length (cM)	3653.1	1669.2
Overall avg. spacing (cM)	4.5	2.1
Length of Chr2 (cM)	1196.8	211.3
Max distance between markers of Chr2 (cM)	1001.5	17.9
Length of Chr6 (cM)	1206.8	208.3
Max distance between markers of Chr6 (cM)	1001.5	16
IC278xIC201	***A. oryzae***	**New**
Overall length (cM)	3995.7	2001.3
Overall avg. spacing (cM)	7	3.5
Length of Chr2 (cM)	1271.9	276.4
Max distance between markers of Chr2 (cM)	1001.5	29.1
Length of Chr6 (cM)	1242.5	243.7
Max distance between markers of Chr6 (cM)	1001.5	19

Figure 4.1 Parental genotype frequencies of progeny strains

Progeny strains positioned arbitrarily based on strain number along the x-axis against frequency of marker calls associated with alleles from the *MAT1-1* parent along the y-axis after filtering. A) IC244 × IC1179 cross showing frequency of IC244 alleles in progeny. B) IC308 × IC307 cross showing frequency of IC308 alleles in progeny. C) IC278 × IC201 cross showing frequency of IC278 alleles in progeny.


A. IC244 alleles
Genotype frequency
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
Index


B. IC308 alleles
Genotype frequency
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
Index

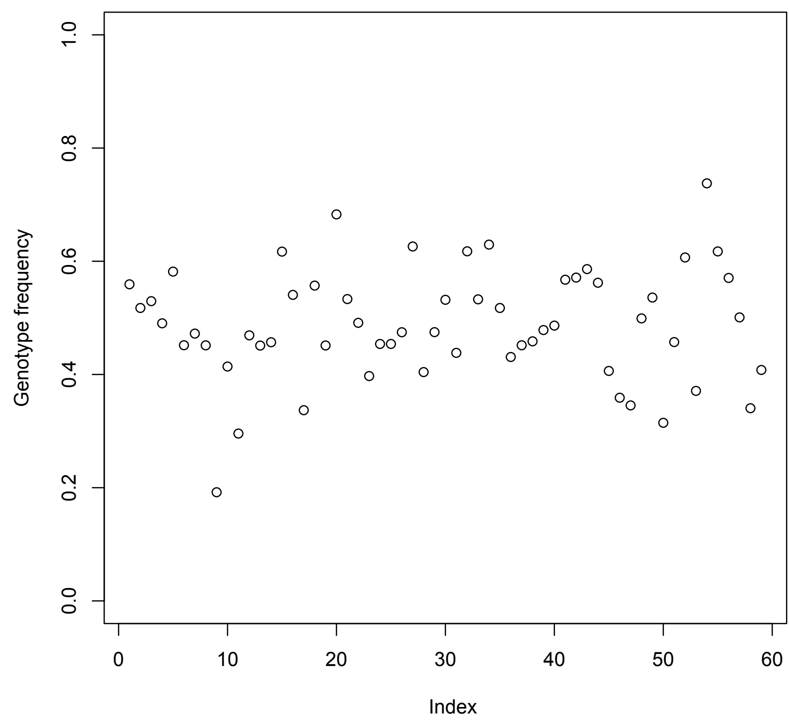
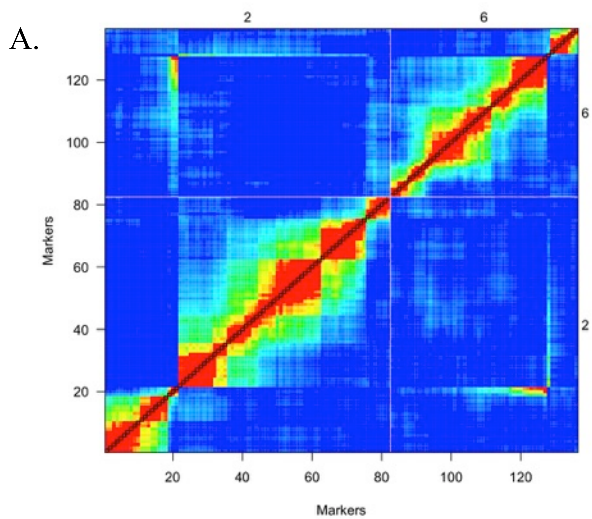

C. IC278 alleles
Genotype frequency
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
Index

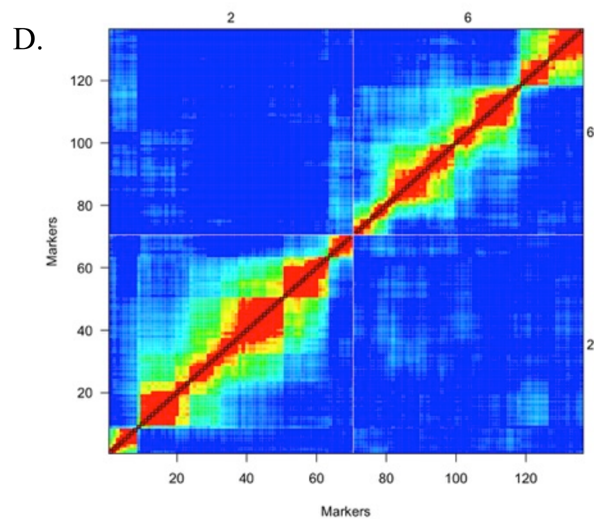
Figure 4.2 Comparison of pairwise recombination fractions and logarithm of the odds (LOD) scores for Chromosome 2 and Chromosome 6 of each cross

Heat-maps where the upper triangle shows recombination fractions between each marker with red values being closer to zero and the bottom triangle shows the LOD score for each of those relationships with higher LOD scores in red. Markers associated with Chromosome 2 and Chromosome 6 ordered based on their positions in the RIB40 reference genome for A) IC244 × IC1179 cross, B) IC308 × IC307 cross, C) IC278 × IC201 cross. The same markers after re-arrangement based on linkage association for D) IC244 × IC1179 cross, E) IC308 × IC307 cross, F) IC278 × IC201 cross.

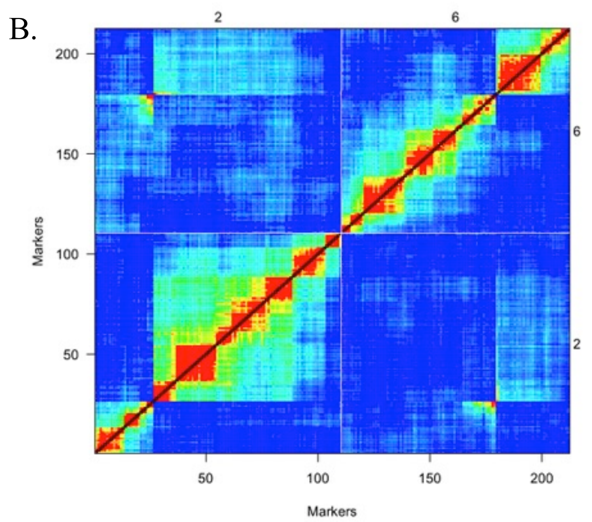
A.



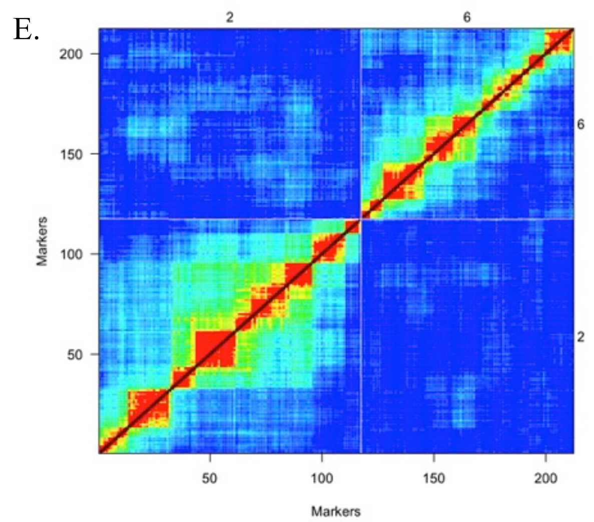
D.



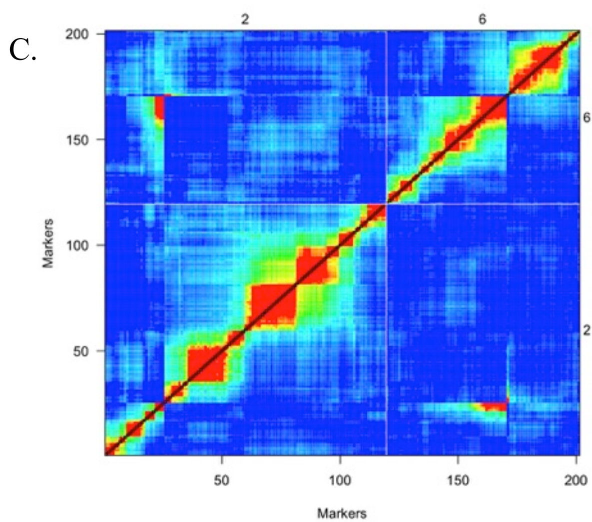
B.



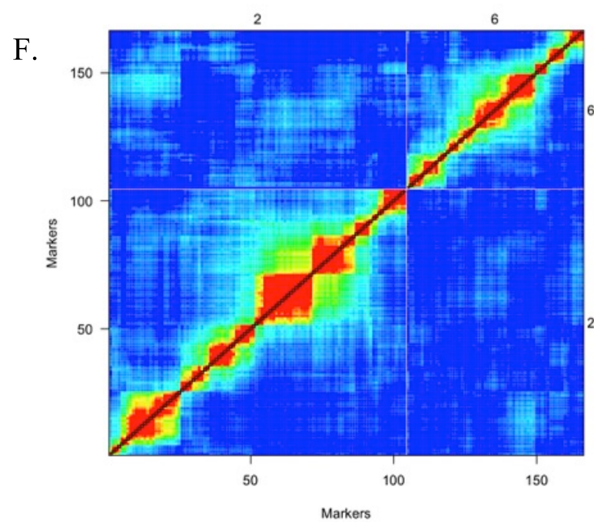
E.



C.



F.



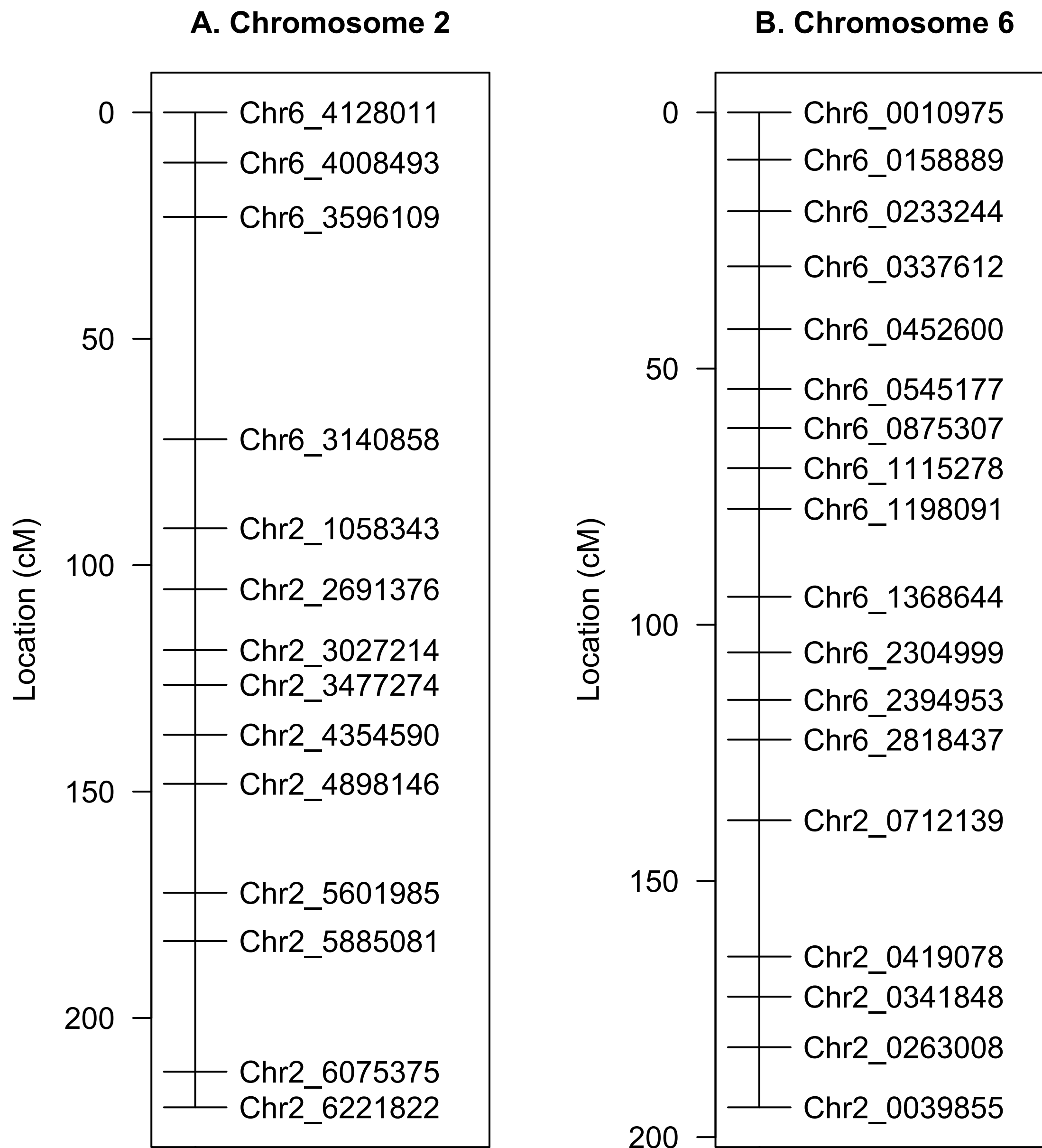
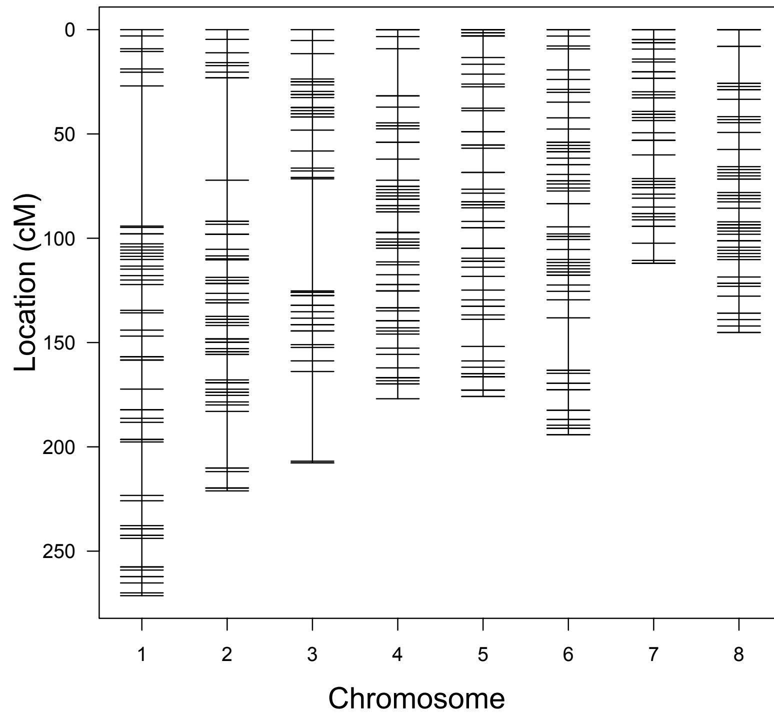


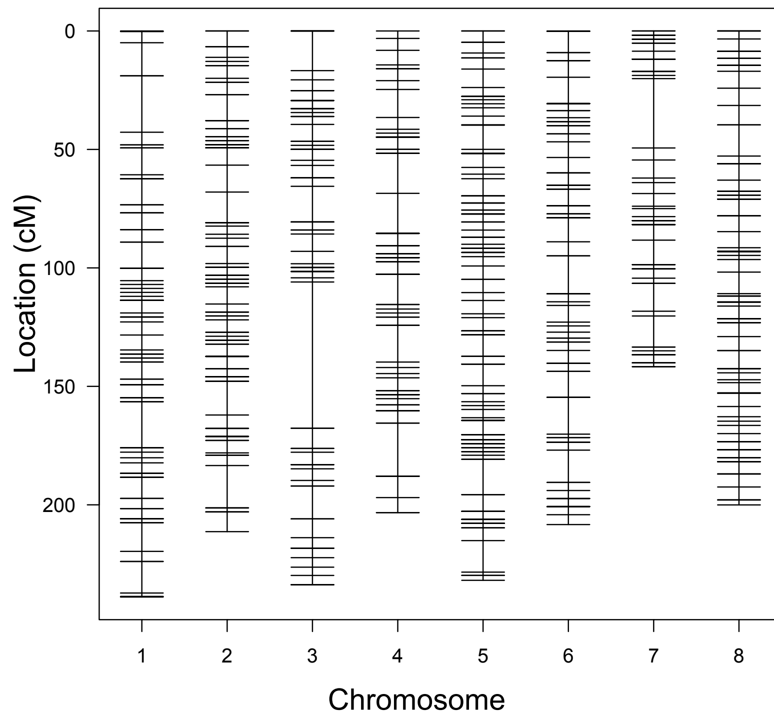
Figure 4.3 Genetic maps of chromosomes involved in inverted reciprocal translocation

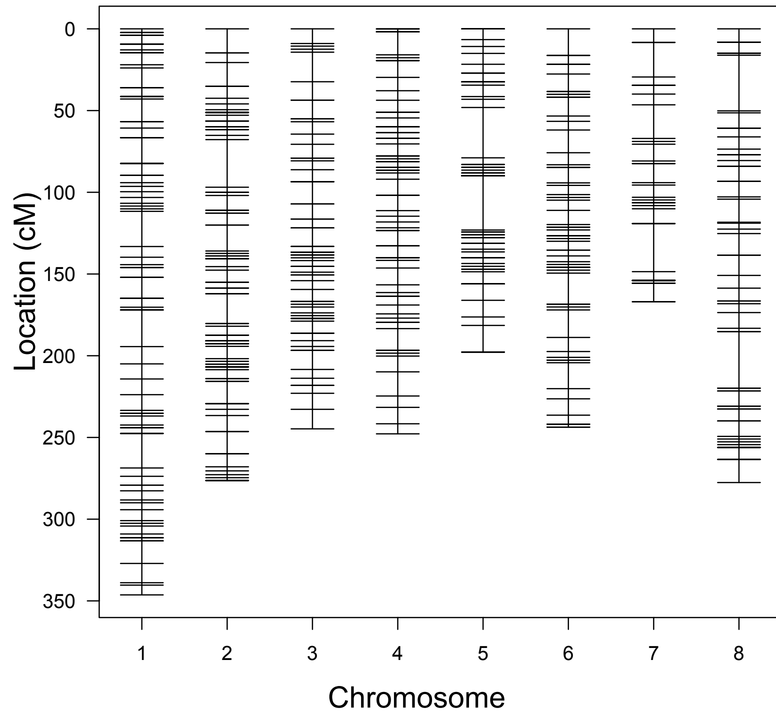
Maps representing the relative position of markers spaced a minimum of 7.5 cM apart in *Aspergillus flavus* based on genetic linkage to show the new organization of markers. Marker names derived from alignment positions in the RIB40 reference genome. A) Chromosome 2 of the IC244 × IC1179 cross. B) Chromosome 6 of the IC244 × IC1179 cross.

Figure 4.4 Genetic maps showing marker distribution

Each marker is positioned along its associated chromosome based on the degree of genetic linkage to the other markers found on the same chromosome. In 100 cM, there is expected to be an average of one recombination event having occurred in a single generation. A) IC244 × IC1179 cross, B) IC308 × IC307 cross, C) IC278 × IC201 cross.


A. IC244 × IC1179
Location (cM)
0
50
100
150
200
250
1
2
3
4
5
6
7
8
Chromosome

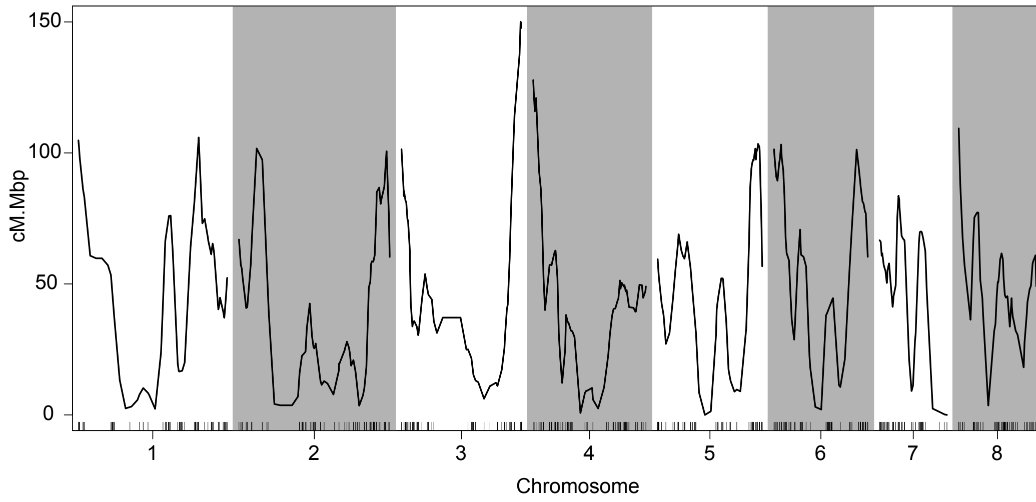

B. IC308 × IC307
Location (cM)
0
50
100
150
200
1
2
3
4
5
6
7
8
Chromosome

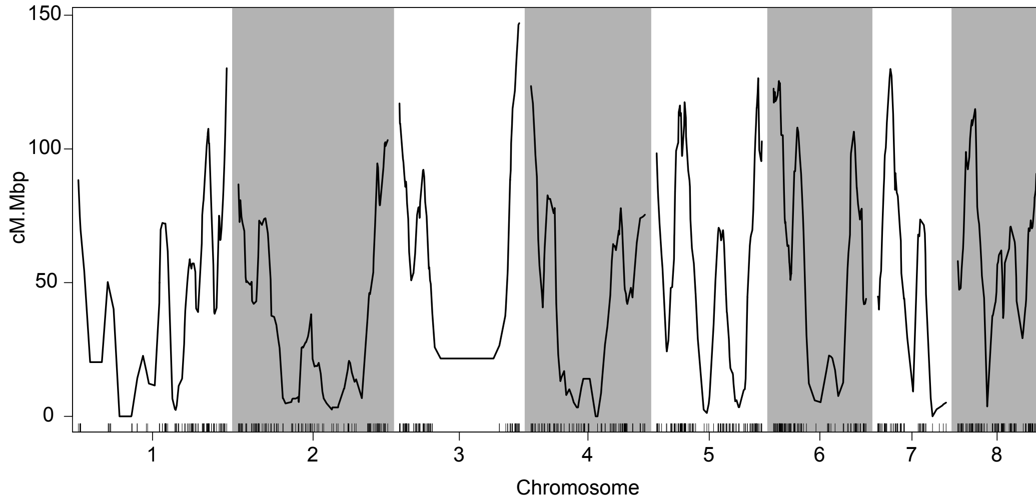


C. IC278 × IC201
Location (cM)
0
50
100
150
200
250
300
350
1
2
3
4
5
6
7
8
Chromosome

Figure 4.5 Recombination rate variation across the genome for each cross

Estimates of recombination rate calculated at each marker position for sliding windows of 500 kb across the reorganized chromosome maps. Distribution of marker positions and chromosome identity is indicated along the x-axis. A) IC244 × IC1179 cross, B) IC308 × IC307 cross, C) IC278 × IC201 cross.


A. IC244 × IC1179
cM.Mbp
150
100
50
0
1
2
3
4
5
6
7
8
Chromosome


B. IC308 × IC307
cM.Mbp
150
100
50
0
1
2
3
4
5
6
7
8
Chromosome

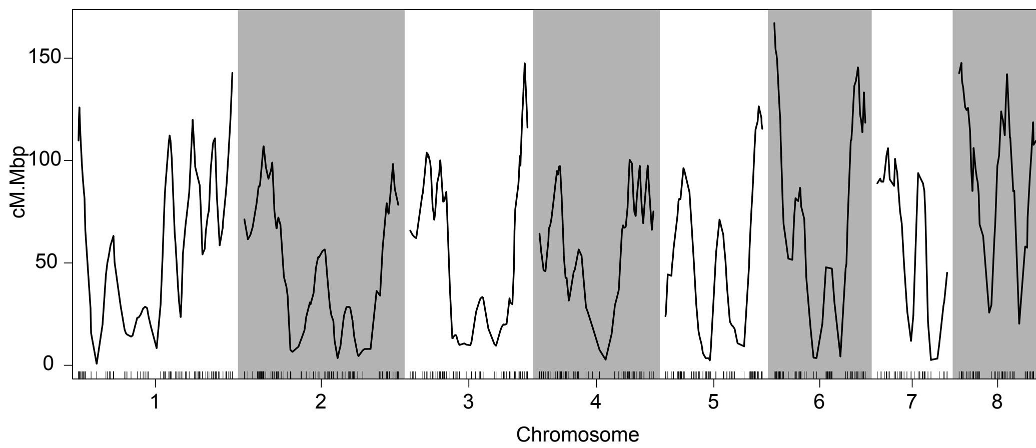
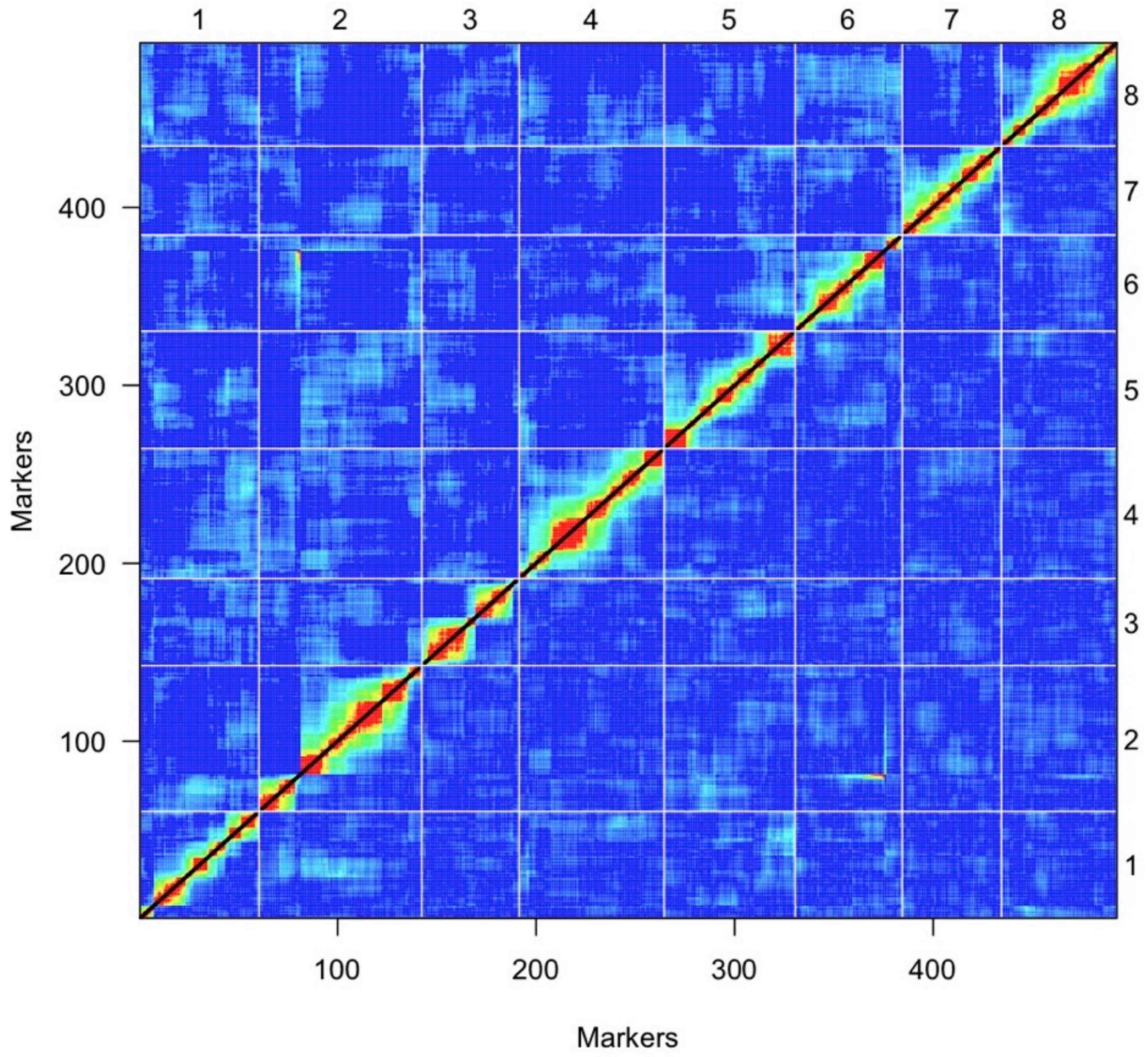

C. IC278 × IC201
cM.Mbp
150
100
50
0
1
2
3
4
5
6
7
8
Chromosome

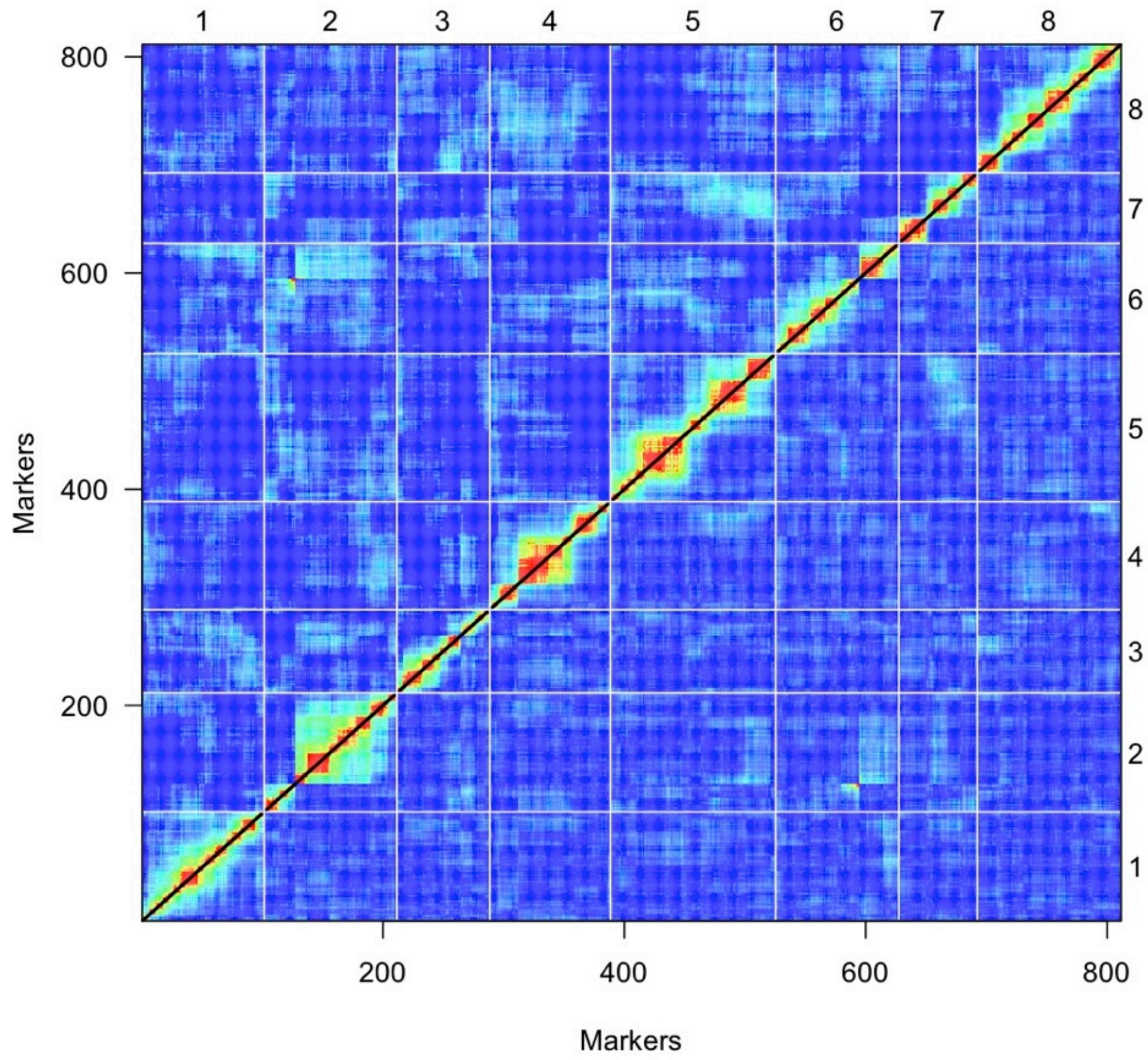
Figure 4.S1 Pairwise recombination fractions and LOD scores for each cross

Heat-maps where the upper triangle shows recombination fractions between each marker with red values being closer to zero and the bottom triangle shows the LOD score for each of those relationships with higher LOD scores in red. Chromosomes along the top and right axis and markers along the left and bottom axis ordered based on the RIB40 reference genome. A) IC244 × IC1179 cross, B) IC308 × IC307 cross, C) IC278 × IC201 cross

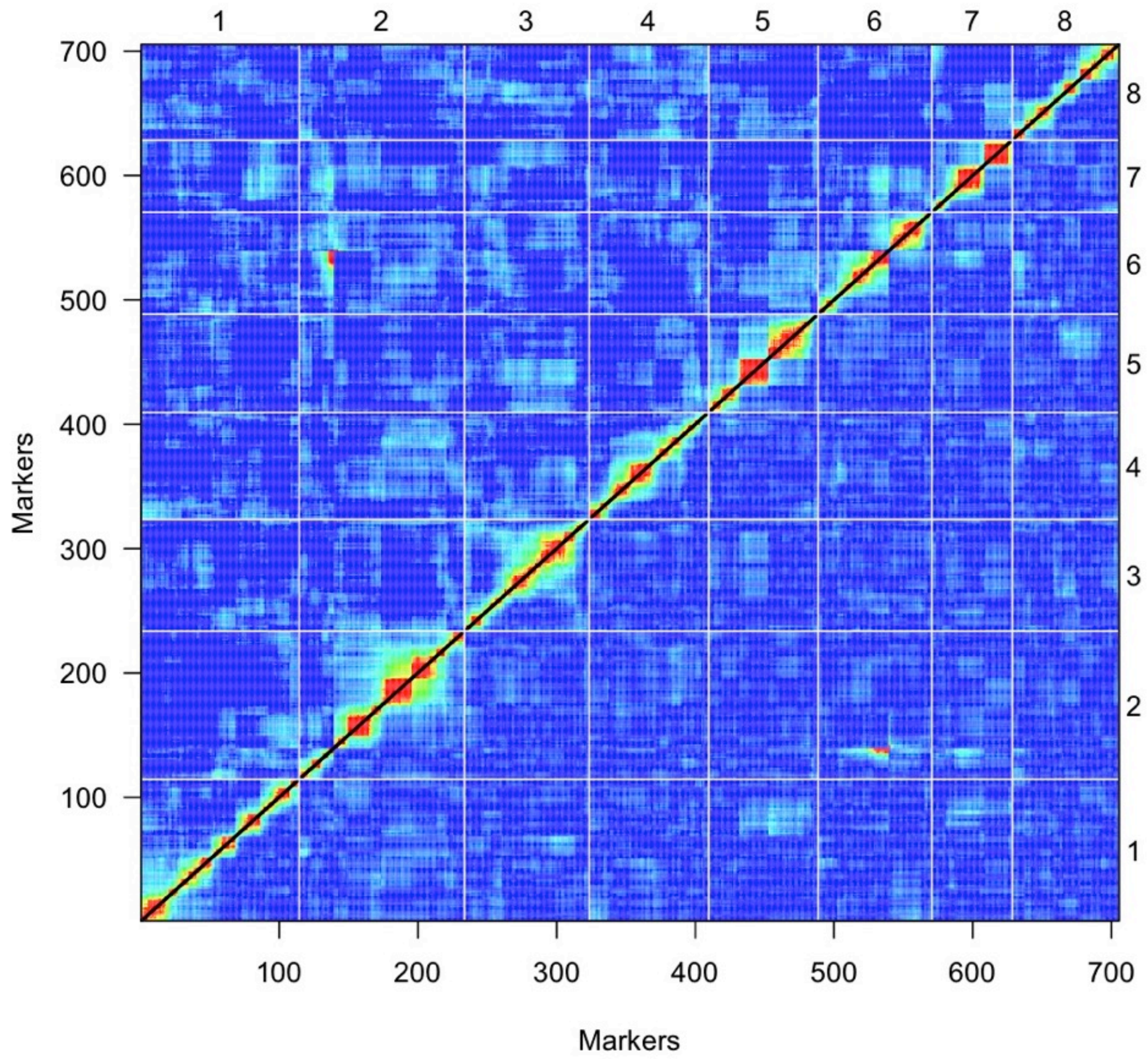
A. IC244 × IC1179



B. IC308 × IC307



C. IC278 × IC201



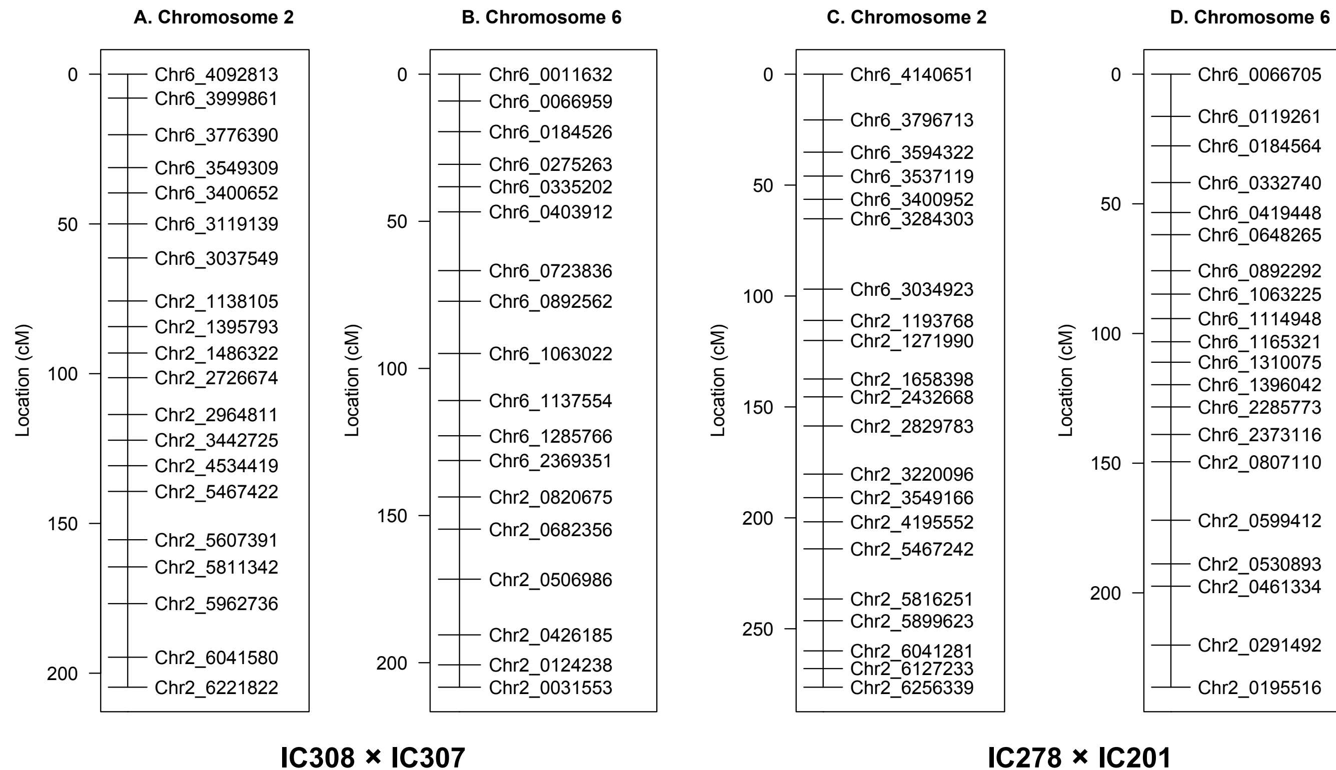


Figure 4.S2 Genetic maps of chromosomes involved in inverted translocation

Maps representing the relative position of markers spaced a minimum of 7.5 cM apart in *Aspergillus flavus* based on genetic linkage to show the new organization of markers. Marker names come from alignment positions in the RIB40 reference genome. A) Chromosome 2 of the IC308 × IC307 cross. B) Chromosome 6 of the IC308 × IC307 cross. C) Chromosome 2 of the IC278 × IC201 cross. D) Chromosome 6 of the IC278 × IC201 cross