

ABSTRACT

OLARTE, RODRIGO AGDUYENG. Population Dynamics of Intra- and Inter-Specific Crosses and the Effect of Biocontrol on Natural Populations of *Aspergillus* species. (Under the direction of Dr. Ignazio Carbone).

My dissertation research evaluates the ramifications of sex in experimental and natural populations of *Aspergillus flavus*. Experimental crosses were performed in the lab and progeny isolates evaluated for toxin heritability and diversity. I used this knowledge to understand population responses to biocontrol application in a longitudinal sampling design in a field in North Carolina.

In my first project, I examined the genotypes of F1 offspring from several experimental crosses of *A. flavus*. Linked loci within the aflatoxin gene cluster on chromosome 3 and unlinked loci on different chromosomes were analyzed to detect crossovers and independent assortment. My results indicate that recombination increases the effective population sizes of aflatoxigenic fungi and is driving genetic and functional hyperdiversity in *A. flavus*. I also observed non-mendelian inheritance of extra-genomic aflatoxin cluster alleles in crosses with at least one parent carrying a partial aflatoxin cluster, which suggests a possible role of cryptic alleles, in addition to sexual recombination, in modulating aflatoxin production.

Aflatoxin production is maintained under balancing selection, however is associated with a cost to production as evidenced by the success of non-aflatoxigenic biocontrol strains used as strategies to reduce aflatoxin contamination in agriculture. These biocontrol strains are applied at high densities to agricultural fields, where they competitively exclude native

aflatoxigenic strains from crops and thereby reduce aflatoxin content. These methods are the most effective means of decreasing aflatoxin contamination in the short-term (*i.e.*, several months); however, usage of biocontrol strains is not sustainable and requires repeat applications yearly to remain effective. Furthermore, the effect of these biocontrol strains on the genetic structure of *A. flavus* populations in these fields is unknown.

In my second project, I sampled *A. flavus* strains from a cornfield in Rocky Mount, NC to determine the effects of using biocontrol strains on the population diversity of *A. flavus*. Soil samples were taken before the application of biocontrol and one year afterward. In addition to the soil samples, sclerotia (sexual structures) were harvested from infected corn ears. Eighty *A. flavus* isolates were collected from the two sets of soil samples and ninety single-ascospore (sexual propagule) isolates were isolated from sclerotia originating from plots treated with biocontrols, for a grand total of 250 isolates. PCR amplification revealed grouping of isolates into three distinct mating-type classes: *MAT1-1*, *MAT1-2* and *MAT1-1/MAT1-2*. A significant proportion of isolates sampled prior to biocontrol treatments were heterokaryotic for mating type (*MAT1-1/MAT1-2*), and this same genotype was found in the ascospore isolates. The vertical transmission of *MAT1-1/MAT1-2* to progeny ascospore isolates suggests that heterokaryosis can be maintained in subsequent generations, and just like in my first project, is driving genetic diversity in *A. flavus*. Furthermore, matings were performed to determine functionality of these *MAT1-1/MAT1-2* strains and all isolates tested were strictly functional as *MAT1-2*, which indicates a non-functional cryptic copy of the mating-type allele. Two biocontrol products are currently approved for commercial application; the population genetic structure before biocontrol application showed the

presence of one biocontrol strain and no detection of the other. One year after the application of the two biocontrol strains, the population genetic structure showed a dominance of the biocontrol type found in the field prior to application. These results indicate the degree of relatedness of the biocontrol strain to the predominant indigenous lineage influences the long-term success of a biocontrol strain. These findings will be instrumental in the selection of a strain for use in next-generation biocontrol strategies.

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Population Dynamics of Intra- and Inter-Specific Crosses and the Effect of Biocontrol on
Natural Populations of *Aspergillus* Species

by
Rodrigo Agduyeng Olarte

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DEDICATION

For helping me achieve my dreams – to my parents, Rodrigo Sr. and Dionicia Olarte.

BIOGRAPHY

His story begins on the morning of September 16, 1980. Rodrigo Agduyeng Olare Jr. was born to Rodrigo Sr. and Dionicia Olarte in the small barangay of Buncag, of the town Dingras in the province of Ilocos Norte, Philippines. In the winter of 1982, he immigrated to the United States with his mother, father and older brother. The family's first home in the US was in the Navy housing community just outside of the Mare Island Naval base in the city of Vallejo of the great state of California and it was there that Rodrigo and his brother started pre-school and would later continue on at Federal Terrace elementary.

In 1985, Rodrigo's sister was born and a short year later the family purchased their first house just outside the Base; this house was Home for the next twelve years at which point Rodrigo would move thirty minutes away to attend college. During this time, Rodrigo's youngest brother was born. Rodrigo graduated from senior high school and then entered as a part of the freshmen class of 1998 at UC Berkeley.

Rodrigo started out pre-med but he decided to go the grad school route when he volunteered for a medical mission in the Philippines; he found out that being a doctor wasn't exactly the right fit for him! His first lab job was in the mouse genetics laboratory of Dr. William Skarnes in the department of Molecular and cell biology (MCB) at UC Berkeley and this is where he would see tails yankin' and heads flyin' (referring to the preferred method of killing a mouse prior to dissection) which made him realize that mammalian systems weren't the right fit for him. He then joined the lab of Dr. John Taylor and worked with

microorganisms where there would be no heads flying or tails yanking. In this lab he would help describe a new species of Archea, *Sulfolobus islandicus*.

He graduated in 2003 with a B.A. in MCB and a minor in the Practice of Art. He then moved on to join the lab of Dr. Matteo Garbelotto where he worked as a Research Associate studying *Phytophthora* and this is where he got his first introduction to the world of plant pathology. In this lab he discovered his passion for the subject and he became so eager to enrich his knowledge in the discipline that he applied to the Plant Pathology program at Cornell University. He spent two years at this prestigious university and completed a Master's degree in 2007 with Drs. Keith Perry and Stewart Gray working on the transmission efficiency of PVY.

After a short stint working as a lab manager with Dr. Terrence Delaney at the University of Vermont, Rod left the cold Northeast and ventured down south to North Carolina, which is where he enrolled in a PhD program to work on *Aspergillus* species. The following is a culmination of six years of hard work and dedication.

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First and foremost I would like to thank the people for whom this dissertation is dedicated to – Mom and Dad thank for your support all throughout the years. You put me through school and always encouraged me to work hard and never give up. The rest of the family, Ron, Jenny and Robert – thanks for keeping me grounded and keeping me on the right path. We are all grown up now and have our own separate lives in different parts of the country, but we'll always be siblings and we'll always be just a phone call or a text message away.

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learned from each other. I also want to thank all the folks in the other CIFR labs – thanks for letting me borrow a lot of lab equipment and chemicals.

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CHAPTER 1

INTRODUCTION: INSIGHTS INTO SEXUAL REPRODUCTION IN *ASPERGILLUS*
FLAVUS

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Abstract

Aspergillus flavus is a heterothallic ascomycete fungus that infects both plants and animals, and is a fungus of toxicological importance due to the production of carcinogenic aflatoxins and other mycotoxins. *A. flavus* is the major producer of AFs in agronomically important crops worldwide. Recent efforts to reduce AF concentrations in grains have focused on the use of two nonaflatoxigenic *A. flavus* strains, AF36 (= NRRL 18543) and NRRL 21882 (the active component of aflu-guard®), as biological control agents. These biocontrol strains are applied at high densities to agricultural fields, where they competitively exclude native aflatoxigenic strains from crops and thereby reduce AF contamination. Although this is reported as an effective method for AF control, the extent to which these biocontrol strains recombine with native strains and the overall effect on the fungal populations is unknown. The possibility of gene flow in nature, even at a low level, could contribute to the persistence and further evolution of aflatoxigenic strains and to the evolution of new species. Here, I aim to discuss the importance of *A. flavus* to agriculture and to human health; next, I will further examine biocontrol as a means of managing AF producing pathogens; and finally, I will present evidence to scrutinize the long-term stability of using such methods.

Role as a plant pathogen

Species in *Aspergillus* section *Flavi* commonly infect agricultural staples such as corn, peanut, cottonseed, and tree nuts and produce an array of mycotoxins (Horn, 2007); the most potent of these are aflatoxins (AFs), which are carcinogenic polyketides. Generally, *Aspergillus flavus* survives as a saprophyte that is able to live in soil and organic debris associated with plant residues (Abbas *et al.*, 2009). *A. flavus* may overwinter as mycelium or as hyphal structures known as sclerotia. The sclerotia can either germinate to produce more hyphae or they may produce conidiospores, which are asexual spores that can be dispersed by air currents or insect vectors and serve as primary inoculum. Unlike most fungi, *A. flavus* favors hot and dry conditions. These fungi are especially problematic in areas that exhibit high amounts of drought (Payne, 1998). During the growing season, infected plant tissues may then serve as sources of secondary inoculum, which can then go on to colonize new non-infected plant tissues.

Aspergillus spp. can either be toxigenic and produce any of a variety of mycotoxins or be nontoxigenic in which toxin production has somehow been impaired; here forward, isolates that are able to produce AFs will be referred to as being aflatoxigenic. Generally, the frequency of aflatoxigenic isolates in nature can range from 50-80%; the relative distribution depends on factors such as the types of plant species present, soil composition, cropping history, crop management, and environmental conditions (Abbas *et al.*, 2009). AF contamination is the basis for yield and monetary losses, not because the fungus drastically hinders the biological growth of its colonized hosts, but because fields are required to be plowed over and completely destroyed when the AF levels within a field are found to reach

or exceed the respective national maximum tolerated level for AFs in human or animal foods (Wu, 2004).

Mycotoxin regulation in agriculture

Several nations have implicated strict standards in regards to maximum tolerated AF contamination levels. For example, the US permits up to 20 µg/kg in food for human consumption and upwards to 300 µg/kg in food for cattle consumption. In China, Guatemala, and Kenya the regulations in food for human consumption are the same as the US, but in Australia, the maximum tolerated level for AFs in human food is 5 µg/kg. In the European Union, the maximum tolerated level is only 4 µg/kg (Wu, 2004). In the US, mycotoxins have been estimated to cause agricultural losses totaling upwards of \$1.4 billion annually, with AF contamination in peanut export worldwide potentially accounting for as much as \$450 million (Wu, 2004; Wu *et al.*, 2008). Robens and Cardwell calculated the costs for AF testing in the US alone to be roughly \$30-\$50 million (Robens, Cardwell, 2003), and the cost grows exponentially when management regimes are implemented (Wu *et al.*, 2008). The need for these regulations is important because AFs can be very detrimental to animals and humans.

Affects on animals and humans

In 1974, AF poisoning epidemic resulted in 106 human deaths in western India (Krishnamachari *et al.*, 1975), and more recently, in 2004, caused the largest death toll from any isolated contamination event killing 125 people in Kenya, East Africa (Giesecker *et al.*,

2004; Probst *et al.*, 2007). AFs have been accused of increasing liver cancer rates and stunting the growths of children in Africa, Southeast Asia, and China (Normile, 2010). In addition to AFs, *A. flavus* produces another unrelated mycotoxin, cyclopiazonic acid (CPA), an indol-tetramic acid that targets the liver, kidneys and gastrointestinal tract in animals (Burdock, Flamm, 2000). AFs and CPA often co-contaminate agricultural products, and several of the symptoms associated with turkey “X” disease in poults, which led to the discovery of AFs in the early 1960s, can be attributed to CPA (Cole, 1986). *Aspergillus fumigatus* is the leading cause of invasive aspergillosis (any of a wide variety of human diseases caused by *Aspergillus spp.*) (Denning, 1998; Hedayati *et al.*, 2007); however, several cases have found *A. flavus* as the more common culprit (Iwen *et al.*, 1997; Talbot *et al.*, 1991). AF B₁ is the most toxic of the AFs and can cause adverse effects in chickens, such as liver damage, impaired productivity and reproductive efficiency, decreased egg quality, and increased susceptibility to disease. In cattle, AF B₁ can lead to reduced weight gain, liver and kidney damage, and a reduction in milk production (Wu, 2004).

AF biosynthesis is regulated by approximately 25 enzymes encoded by genes that are clustered together in a 70-kb telomeric region of the right arm of chromosome 3 (Yu *et al.*, 2004). CPA involves approximately 3-5 enzymes encoded in a 50-kb mini-cluster adjacent to the AF gene cluster (Chang *et al.*, 2009). The inability to produce AFs or CPA in *A. flavus* is often due to various deletions in these gene clusters (Chang *et al.*, 2005; Chang *et al.*, 2009). Populations of *A. flavus* show a high level of variation in mycotoxin production, with individuals producing both AFs and CPA, AFs alone, CPA alone or neither mycotoxin (Horn, Dorner, 1999; Horn *et al.*, 1996). Recent analysis of molecular genetic variation in

the AF gene cluster in a field population of *A. flavus* has revealed distinct recombination blocks and a hotspot (region with an elevated number of recombination events) that coincides with genes lost in deletion strains (Moore *et al.*, 2009). Gene loss in the AF gene cluster is not always accompanied by gene loss in the adjacent CPA cluster and in at least one deletion strain (IC311) CPA is still produced (Moore *et al.*, 2009). However, most AF deletion strains do not make CPA, as a result of the deletion of one or more genes important in CPA biosynthesis; some strains have larger deletions upstream of the CPA cluster such that the telomere is immediately adjacent to genes flanked by recombination blocks (e.g., *aflM* in IC313 (Moore *et al.*, 2009)). It appears that partial clusters align with recombination block boundaries, which suggests that recombination might account for much of the variation in CPA and AF production observed in populations. Control of these aflatoxigenic strains of *A. flavus* has relied on the usage of non-aflatoxigenic isolates and the notion that higher amounts of the nonaflatoxigenic isolates will be able to outcompete the aflatoxigenic ones.

Biological control

Two biocontrol strains, AF36 and NRRL21882, are currently approved by the Environmental Protection Agency (EPA) for use in agricultural management. AF36 was originally isolated from a cotton field in Arizona (EPA, 2003) and approved for commercial use on cotton in Arizona and Texas. Ehrlich and Cotty reported that loss of aflatoxigenicity in AF36 is the result of a nonsense mutation in *pksA* (*aflC*), a critical early pathway gene in AF biosynthesis (Ehrlich, Cotty, 2004). AF36 has otherwise a full AF gene cluster and a functional CPA cluster. NRRL 21882 was isolated from a peanut seed in Georgia (EPA, 2004) and is

currently approved for use on peanuts and corn in the US. Chang et al. (Chang *et al.*, 2005) first reported that NRRL 21882 is missing the entire AF and CPA gene clusters. The commercial application of highly competitive aggressive non-aflatoxigenic strains of *A. flavus* has been shown to be effective in reducing AF contamination in peanuts (Dorner *et al.*, 1992; Dorner *et al.*, 1998), corn (Dorner *et al.*, 1999) and cottonseed (Cotty, 1994; Cotty, Bhatnagar, 1994). Biological control is currently the most effective means of reducing inoculum levels of AF-producing fungal species in agricultural systems (Abbas *et al.*, 2009); however, if genetic exchange were found to occur within these fungal populations, the stability of such practices may be questioned.

Discovery of the Sexual Cycle

The possibility that recombination in *A. flavus* could potentially influence the stability of biocontrol strains was first suggested by David Geiser, who reported a population structure in *A. flavus* indicative of recombination based on a lack of congruence of five gene genealogies (Geiser *et al.*, 1998); however, the method of recombination, how often recombination occurs, or when recombination occurred in the history of the species could not be determined. Similar evidence for recombination based on noncongruence of gene genealogies for four genes was shown for *Aspergillus nomius* (Peterson *et al.*, 2001), another AF-producing species. More recently Carbone *et al.* sequenced 21 intergenic regions of the AF gene cluster in strains from an *Aspergillus parasiticus* population in a Georgia peanut field (Carbone *et al.*, 2007). The population had been previously characterized according to morphology, mycotoxin production, and vegetative compatibility (Glass *et al.*, 2000; Leslie,

1993). While coalescent analysis suggested that some recombination had occurred within the last one million years, the authors also found that recombination alone separated some AF cluster haplotypes, indicating that recombination could be recent.

Asexual reproduction is the most common method by which these fungi reproduce. The most distinctive feature of the Aspergilli is the asexual structure known as the conidiophore, which is borne on the mycelial threads and gives rise to conidiospores (Fig. 1) (Casselton, Zolan, 2002). Each conidiospore has the ability to disperse and germinate into a mycelium that is then able to undergo another cycle of asexual reproduction. Prior to 2009, *A. flavus* was known as an imperfect fungus, meaning the sexual cycle was not known to exist. Although genealogical approaches have provided indirect evidence of recombination, in 2009, Horn et al. (Horn *et al.*, 2009) provided the first direct evidence that sexual recombination may be an important mechanism for generating diversity in mycotoxin production in these agriculturally important species. The authors described *Petromyces flavus*, the sexual state of *A. flavus*, from crosses between strains of the opposite mating type (Horn *et al.*, 2009). They demonstrated that sexual reproduction in *A. flavus* is heterothallic and occurs between individuals belonging to different vegetative compatibility groups, which suggest that the vegetative compatibility system is not a barrier to gene flow.

In addition to the sexual compatibility system, a second compatibility system controls the stable fusion of hyphae. Anastomoses bring together different nuclei within the same hyphal cells, producing a heterokaryon (Fig. 1) (Casselton, Zolan, 2002). This vegetative compatibility system is dictated by a series of heterokaryon incompatibility (*het*) loci whose alleles must all be identical for stable hyphal fusions to occur (Glass *et al.*, 2000; Leslie,

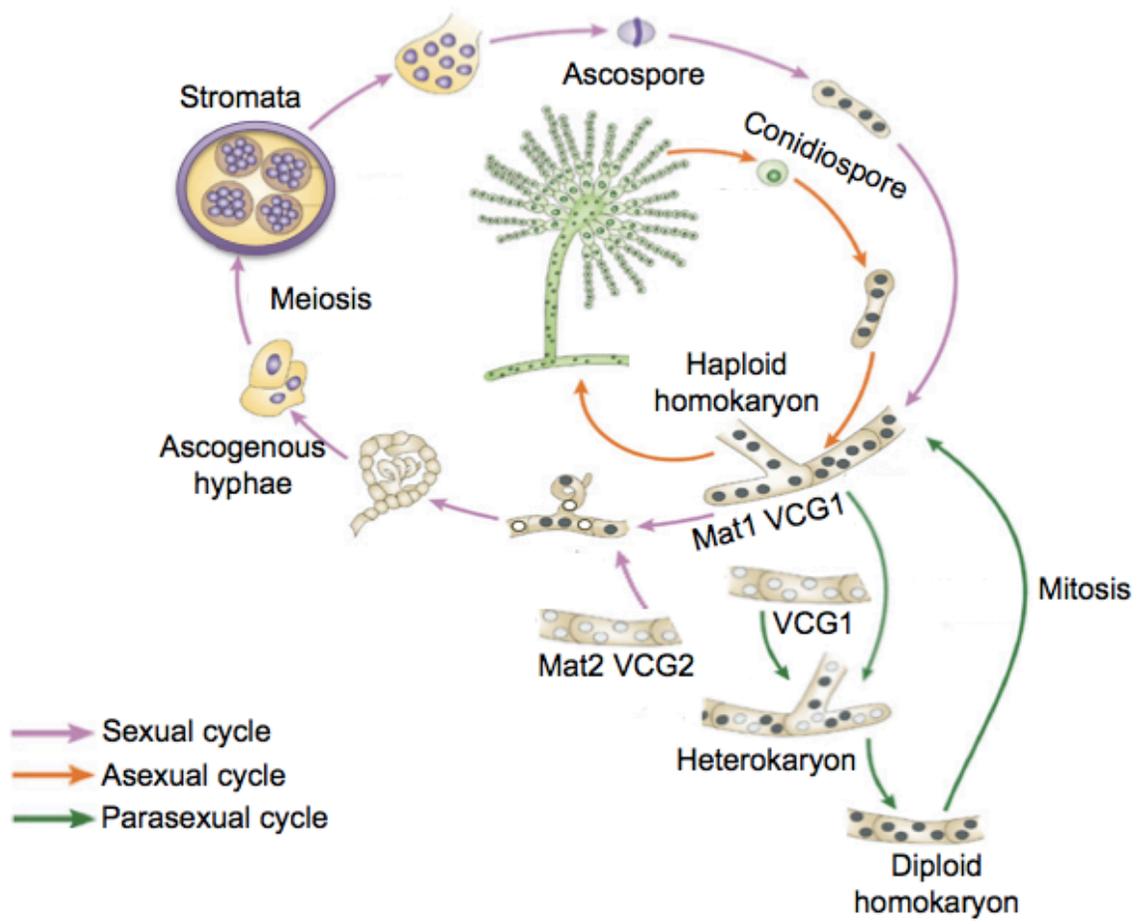
1993). The number of *het* loci in *A. flavus* has not been determined, but in *A. nidulans*, a minimum of eight *het* loci are distributed over five linkage groups (Anwar *et al.*, 1993). Vegetatively compatible individuals within populations are often grouped together into vegetative compatibility groups (VCGs) and provide a multilocus measure of genetic diversity (Horn, Greene, 1995). Crosses between vegetatively incompatible individuals trigger a programmed cell death reaction characterized by severe growth inhibition, repression of asexual sporulation, hyphal compartmentation and apoptotic-type death in the heterokaryotic cell (Glass *et al.*, 2000). Heterokaryon incompatibility is thought to act as a self/non-self recognition system that prevents hyphal fusion and genetic exchange between strains that carry different alleles at *het* loci. Most of the variation in AF production within populations is due to differences among VCGs, with little variation occurring among strains within individual groups (Horn *et al.*, 1996).

The similarity in AF profiles for all strains within a VCG suggests that some degree of clonal amplification is occurring in nature (Ehrlich *et al.*, 2007). Within a single VCG, genetic exchange and recombination is also possible through parasexuality, a mechanism whereby the nuclei of heterokaryons fuse and shuffle genetic material (Fig. 1) (Casselton, Zolan, 2002). Although parasexuality has yet to be demonstrated conclusively in nature, mitotic recombination through parasexuality has been demonstrated in *A. flavus* and *A. parasiticus* under controlled laboratory conditions (Papa, 1973; Papa, 1978).

Implications of genetic exchange

Major evolutionary advantages of recombination include: 1) the production of novel

Figure 1.1. Adapted from Casselton, Zolan, 2002. The reproductive life cycles of *Aspergillus flavus*. *A. flavus* is heterothallic, meaning sexual reproduction requires the fusion between hyphae of opposite mating types. Ascogenous hyphae, after meiosis, results in the formation of a stromata, which houses ascospores. Ascospores may disperse once the stromata is opened and can then go on and germinate into a mycelium. The asexual cycle is distinguished by the conidiophore, which gives rise to conidiospores. Each conidiospore may germinate into a mycelium, which can give rise to more conidiophores and the cycle may then be repeated. The parasexual cycle occurs between individuals belonging to the same vegetative compatibility group. The formation of a heterokaryon ensues after a successful fusion between compatible hyphae. Haploidization is achieved through mitosis.



genotypes that may allow organisms to adapt quickly to changing environments, 2) advantageous gene combinations can be formed more rapidly by recombination than in asexual populations, which can only produce novel genotypes by successive mutations, and 3) recombination is a way of purging deleterious mutations that would otherwise accumulate in asexual clones (Milgroom, 1996). An example that demonstrates these principles comes from a study in which several strains of a fungal pathogen (*Mycosphaerella graminicola*) were inoculated onto moderately resistant and susceptible cultivars of wheat (Zhan *et al.*, 2007). After a time span of 60 days, samples of the fungus were collected and genetically analyzed. The authors found that recombinants were recovered at a higher frequency on the moderately resistant host plant compared to the susceptible host plant. They also found that these recombinant pathogen strains displayed higher levels of fitness, virulence and fungicide tolerance. Moreover, they concluded that sexual reproduction facilitates the evolution of parasites in overcoming host resistance. This study exemplifies the evolutionary roles of sexual and asexual reproduction. Sex enables a fungal pathogen to generate progeny that are able to survive in antagonistic environments (in this case referring to the moderately resistant host plant), whereas asexual reproduction maintains pathogen population levels in favorable environments. Ultimately, the genetic diversity of these pathogens have changed and if allowed to continue evolving, the effective pathogen population sizes of the more fit, more virulent, and more fungicide tolerant individuals would continue to increase.

Another example to suggest that sex in fungal or fungal-like organisms leading to new and potentially more problematic pathogen populations comes from oomycetes or fungal-like organisms. Hybridization is possible in the *Phytophthoras* and is why great

measures are being expended to keep opposite mating types of respective *Phytophthora* species from interacting with each other in the wild. Hybridization between different species of *Phytophthoras* can produce a new species with different properties from those of either parent. In Europe, a new *Phytophthora* emerged in 1993 that began to attack alder trees; this new species was later classified as a product of a sexual recombination event between *Phytophthora cambivora* and an unknown species similar to *Phytophthora fragariae*, neither of which are known to infect alder (Brasier *et al.*, 1999). This example supports the idea that sexual reproduction leads to greater genetic diversity in the populations of these pathogens. In this case, a specific combination of genes were mixed together creating a new species thereby expanding the host range of these pathogens.

McDonald and Linde inferred the evolutionary potential of a pathogen on the basis of the combined effects of five evolutionary forces, including: mutation, genetic drift, gene flow, reproduction/mating system, and selection (McDonald, Linde, 2002). A scale of evolutionary risk was developed in which a pathogen could be ranked on a scale from 1 to 9, with 1 being the lowest risk and 9 being the highest risk. This model assumes large population sizes, constant mutation rates, and that selection is efficient for all pathogens, therefore only assesses a pathogen's evolutionary risk according to its reproduction/mating system and gene flow potential. Using the attributes of this scale for *A. flavus*, prior to knowledge regarding sexual reproduction in this fungus, *A. flavus* would have been given a rank order of 3, which is on the lower end of the scale; however, with the existence of a sexual reproductive cycle, *A. flavus* leaps from an evolutionary risk distinction of 3 to the

maximum value of 9. The overall value of evolutionary risk represents the amount of variability possible in a population of the respective pathogen.

A large factor in the approval of the biocontrol strains afore mentioned for use in agricultural systems was based on the notion that sexual reproduction was not a part of the life cycles of these pathogens, therefore genetic recombination through sexual reproduction was not considered a possible source of strain instability. Evidence to support that sexual reproduction leads to a population dynamic in which highly toxigenic strains are favored comes from the aflatoxigenic profiles of *A. flavus* populations from Argentina (South America) and Georgia (North America) (Moore unpublished). The Argentinean population represents a population with infrequent sexual reproduction as characterized by a disproportionate number of the two mating types; however, the Georgian population has an equal proportion of the mating types. When compared, the overall Georgian population had a higher toxin load than the Argentinean population, suggesting that sexual reproduction favored selection towards aflatoxigenic individuals.

In the usage of biocontrol strains, large amounts of the fungus are deployed, which in essence increases the effective population size of individuals out in the field that are able to reproduce and recombine to further diversify the genetic makeup of the pathogen populations. The production of progeny that are more fit or are able to produce greater amounts of AFs compared to the original population may be probable as evolution and sexual reproduction continue to happen. Instead of biocontrol, which increases the numbers of individuals within the fungal populations, other methods of control that reduces inoculum levels may be safer in the long term. Biocontrol, however, may still be an effective approach

if a biocontrol strain is found that somehow not only reduces the population size of aflatoxigenic strains but also reduces the effective population sizes of the fungal pathogen overall. Several questions that will be addressed in future work include: what is the heritability of these mycotoxins?, does fecundity vary among strains of *A. flavus*?, is sex happening in nature?, and what are the genes that regulate vegetative compatibility?

Conclusion

Further research into the biology of the sexual state of *A. flavus* is required to be able to further evaluate the stability and efficacy of the usage of biocontrol strains as a means to combating against aflatoxigenic fungi in agricultural production. Currently, biocontrol is still the most effective means of controlling the associated aflatoxigenic fungal pathogens, however, the stability of such control methods may face scrutiny with proof of gene flow within *A. flavus* populations. The following chapters will address these concerns.

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CHAPTER 2

EFFECT OF SEXUAL RECOMBINATION ON POPULATION DIVERSITY IN AFLATOXIN PRODUCTION BY *ASPERGILLUS FLAVUS* AND EVIDENCE FOR CRYPTIC HETEROKARYOSIS

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Abstract

Aspergillus flavus is the major producer of carcinogenic aflatoxins (AFs) in crops worldwide. Natural populations of *A. flavus* show tremendous variation in AF production, some of which can be attributed to environmental conditions, differential regulation of the AF biosynthetic pathway, and deletions or loss-of-function mutations in the AF gene cluster. Understanding the evolutionary processes that generate genetic diversity in *A. flavus* may also explain quantitative differences in aflatoxigenicity. Several population studies using multilocus genealogical approaches provide indirect evidence of recombination in the genome and specifically in the AF gene cluster. More recently *A. flavus* has been shown to be functionally heterothallic and capable of sexual reproduction in laboratory crosses. In the present study, we characterize the progeny from nine *A. flavus* crosses using toxin phenotype assays, DNA sequence-based markers and array comparative genome hybridization. We show high AF heritability linked to genetic variation in the AF gene cluster, as well as recombination through the independent assortment of chromosomes and through crossing over within the AF cluster that coincides with inferred recombination blocks and hotspots in natural populations. Moreover, the vertical transmission of cryptic alleles indicates that while an *A. flavus* deletion strain is predominantly homokaryotic, it may harbor AF cluster genes at a low copy number. Results from experimental matings indicate that sexual recombination is driving genetic and functional hyperdiversity in *A. flavus*. The results of this study have significant implications for managing AF contamination of crops and for improving biocontrol strategies using non-aflatoxigenic strains of *A. flavus*.

Introduction

Aspergillus flavus commonly infects agricultural staples, such as corn, peanuts, cottonseed and tree nuts, and produces aflatoxins (AFs), which are carcinogenic polyketides that pose a serious health risk to humans worldwide (Bennett, Klich, 2003; Eaton, Groopman, 1994; Squire, 1981; Wu, 2004). In 1974, an AF poisoning epidemic resulted in 106 human deaths in western India (Krishnamachari *et al.*, 1975), and more recently, in 2004, AFs were responsible for 125 fatalities in Kenya, East Africa (Gieseke *et al.*, 2004; Probst *et al.*, 2007). AFs have also been associated with increased liver cancer rates and stunted growth of children in Africa, Southeast Asia, and China (Normile, 2010). In addition to AFs, *A. flavus* produces another unrelated mycotoxin, cyclopiazonic acid (CPA), an indol-tetramic acid that targets the liver, kidneys and gastrointestinal tract in animals (Burdock, Flamm, 2000). AFs and CPA often co-contaminate agricultural products, and several of the symptoms associated with turkey “X” disease in poults, which led to the discovery of AFs in the early 1960s, can be attributed to CPA (Cole, 1986). *A. flavus* is second only to *A. fumigatus* in incidences of human invasive aspergillosis (Denning, 1998; Hedayati *et al.*, 2007; Iwen *et al.*, 1997; Talbot *et al.*, 1991); in a survey of 53 reported outbreaks and 458 affected patients, the most common species implicated were *A. fumigatus* in 154 patients and *A. flavus* in 101 patients (Vonberg, Gastmeier, 2006).

In the United States, mycotoxins are estimated to cause agricultural losses totaling upwards of \$1.66 billion annually (Vardon *et al.*, 2003). AF contamination in peanut exports worldwide potentially accounts for as much as \$450 million (Wu, 2004; Wu *et al.*, 2008). Robens and Cardwell (2003) calculated the costs for AF testing in the United States alone to

be roughly \$30-\$50 million. The costs grow exponentially when management regimes are implemented (Wu *et al.*, 2008). Recent efforts to reduce AF concentrations in crops have focused on the use of two non-aflatoxigenic (AF-) *A. flavus* strains, AF36 (=NRRL 18543) and NRRL 21882 (the active component of Afla-Guard[®]), as biological control agents. These biocontrol strains are applied at high densities to agricultural fields, where they competitively exclude native aflatoxigenic (AF+) strains from crops and thereby reduce AF contamination (Dorner, 2005). AF36 was originally isolated from a cotton field in Arizona and was approved for commercial use on cotton in Arizona and Texas (EPA, 2003). Ehrlich and Cotty (2004) reported that loss of aflatoxigenicity in AF36 is the result of a nonsense mutation in *pksA* (=aflC), a critical early pathway gene in AF biosynthesis. AF36 has otherwise a full AF gene cluster and a functional CPA cluster. Although AF36 is effective in excluding toxigenic strains and reducing AF levels, this strain is reported to significantly increase CPA contamination in food and feed commodities (Abbas *et al.*, 2011). NRRL 21882 was isolated from a peanut seed in Georgia and is currently approved for use on peanuts and corn in the United States (EPA, 2004). Chang *et al.* (2005) first reported that NRRL 21882 is missing the entire AF and CPA gene clusters. The commercial application of highly competitive AF- strains of *A. flavus* has been shown to significantly reduce AF contamination in peanuts (Dorner *et al.*, 1992; Dorner *et al.*, 1998), corn (Dorner *et al.*, 1999) and cottonseed (Cotty, 1994; Cotty, Bhatnagar, 1994). NRRL 21882 may be more effective than AF36 in reducing both AF and CPA levels (Abbas *et al.*, 2011). However, neither biocontrol strain is easy to track after application (Das *et al.*, 2008) and both decline in incidence relative to native strains after initial application. Whether biocontrol strains are at

a selective disadvantage or are assimilated by indigenous strains through genetic exchange and recombination is unknown.

We recently described *Petromyces flavus*, the sexual state of *A. flavus*, from crosses between strains of the opposite mating type (Horn *et al.*, 2009a). We further demonstrated that sexual reproduction in *A. flavus* is heterothallic and occurs between individuals belonging to different vegetative compatibility groups (VCGs) (Horn *et al.*, 2009a; Ramirez-Prado *et al.*, 2008). Vegetative or heterokaryon incompatibility is a self/non-self recognition system whereby compatible hyphal cells with matching heterokaryon incompatibility alleles can fuse and undergo genetic exchange through parasexuality; in contrast, hyphal contact between incompatible genotypes triggers programmed cell death (Leslie, 1993). In *A. flavus* populations, most of the variation in morphology and mycotoxin production can be attributed to differences among VCGs (Horn *et al.*, 1996). Here we examine the role of sexual reproduction in generating mycotoxin diversity in *A. flavus*.

Materials and Methods

Sexual crosses

Nine crosses of sexually compatible *A. flavus* strains were set up following the methods previously described (Horn *et al.*, 2009a; Horn *et al.*, 2009b). All parents were also self-crossed to examine the possibility of self-fertility (see Table 2.1). Strain selection was based on two distinct *A. flavus* lineages, groups IB and IC, described in a previous study (Moore *et al.*, 2009). Group IC includes a mix of AF+ (IC244, IC278, IC301, IC307, IC308) and AF- (AF36) isolates, whereas group IB appears to be strictly AF- and comprises isolates with full

Table 2.1. Incidences of sexual state in *Aspergillus flavus* crosses

<i>MATI-1</i> ¹			<i>MATI-2</i> ¹			Number of sclerotia/stromata per slant ⁴	Number of sclerotia/stromata examined	% with ascocarps ^{4,5}	% with ascospore-bearing ascocarps ^{4,6}
IC strain	NRRL strain ²	VCG ³	IC strain	NRRL strain ²	VCG ³				
Single strains									
IC244	29473	17				427 ± 34	300	3.3 ± 1.1	0
IC278	29507	33				730 ± 53	300	0	0
IC301	29530	56				1213 ± 80	300	0	0
IC308	29537	63				1384 ± 91	300	0	0
IC310	35736	ND ⁷				308 ± 21	300	0	0
IC311	35737	ND				1525 ± 60	300	14.0 ± 3.6	0
			IC277	29506	32	1617 ± 107	300	5.0 ± 2.0	0
			IC307	29536	62	674 ± 35	300	0	0
			IC313	35739	76	132 ± 48	300	0	0
			IC316	21882 ⁸	24	1245 ± 160	300	0.3 ± 0.6	0
			AF36 ⁹	18543	YV36	74 ± 23	291	0	0
Crosses									
AF+ isolates within group IC									
IC308	29537	63	IC307	29536	62	342 ± 2	300	72.7 ± 4.0	50.3 ± 14.0
AF+ and AF- isolates within group IC									
IC278	29507	33	AF36	18543	YV36	62 ± 36	245	79.2 ± 5.9	79.2 ± 5.9
IC301	29530	56	AF36	18543	YV36	103 ± 46	327	8.5 ± 3.5	3.8 ± 1.5

Table 2.1. Continued

AF+ from group IC, and AF- isolates from group IB									
Group IC			Group IB						
IC244	29473	17	IC277	29506	32	604 ± 34	440	8.2 ± 7.0	6.5 ± 4.9
IC244	29473	17	IC316	21882	24	257 ± 78	300	27.0 ± 7.2	26.0 ± 6.1
IC278	29507	33	IC277	29506	32	489 ± 85	300	33.0 ± 4.0	28.7 ± 4.6
IC278	29507	33	IC313	35739	76	47 ± 18	190	62.2 ± 16.9	51.1 ± 14.9
AF- isolates within group IB									
IC310	35736	ND	IC316	21882	24	334 ± 4	300	11.3 ± 3.5	8.0 ± 1.0
IC311	35737	ND	IC277	29506	32	836 ± 41	300	62.0 ± 6.9	36.3 ± 5.5

¹Mating-type designations for NRRL 29473, 29506, 29507, 29530, 29536, 29537 from Ramirez-Prado *et al.* (2008).

²Strain numbers (NRRL) from Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

³Vegetative compatibility groups based on Horn and Greene (1995) and Ehrlich *et al.* (2007).

⁴Means ± s.d. ($n = 3-5$ culture slants).

⁵Percentage of total number of sclerotia/stromata examined containing one or more ascocarps irrespective of the presence of ascospores.

⁶Percentage of total number of sclerotia/stromata examined containing one or more ascospore-bearing ascocarps.

⁷ND = not determined.

⁸NRRL 21882 = Afla-Guard[®] strain.

⁹AF36 = IC1179.

(IC277, IC310), partial (IC311, IC313) or completely missing (NRRL 21882) AF cluster genes. All group IC isolates examined in this study are CPA+, whereas group IB isolates can be classified as CPA+/AF- (IC310, IC311) or CPA-/AF- (IC313, NRRL 21882). Parental strains were selected such that crosses were performed between: (1) AF+ isolates within group IC (IC308 × IC307); (2) AF+ and AF- isolates within group IC (IC278 × AF36, IC301 × AF36); (3) AF+ isolates from group IC and AF- isolates from group IB (IC244 × IC277, IC244 × NRRL 21882, IC278 × IC277, IC278 × IC313); and (4) AF- isolates within group IB (IC310 × NRRL 21882, IC311 × IC277). For each cross, up to 300 sclerotia, when available, were sliced open and examined for ascocarps and ascospores. Single ascospore progeny isolates were obtained from individual ascocarps within stromata according to Horn *et al.* (2009b).

Mycotoxin quantification and heritability

AF and CPA production by parents and F1 progeny were determined by inoculating 1 mL of yeast-extract sucrose broth in three replicate 4-mL vials with dry conidia (approximately 10^5) (Horn, Dorner, 1999). Cultures were incubated for 7 days at 30 °C in darkness. Vial cultures were analyzed by high performance liquid chromatography for production of AFs and CPA as previously described (Horn, Dorner, 1999; Horn *et al.*, 1996). Limits of quantification are 0.5 ng of AF B₁ and 2 µg of CPA per mL of culture medium. In previous work we calculated the heritability of AFs G₁, G₂, B₁, and B₂ in *A. parasiticus* crosses (Horn *et al.*, 2009b) and found that an examination of twelve offspring in each of four different crosses was sufficient for precise heritability estimates (Carbone, unpublished data). We

therefore followed a similar experimental design here with *A. flavus* and examined eleven or twelve offspring for each of the nine crosses. Mycotoxin heritability was estimated as the slope of the line obtained by regressing offspring AF and CPA trait values on the values of the mid-parent.

DNA isolation and multilocus sequencing

Total genomic DNA was extracted from freeze-dried mycelia using either the Qiagen maxiprep kit (Qiagen, Valencia, CA) or the MasterPure Yeast DNA Purification Kit (Epicentre Technologies, Madison, WI) following the manufacturer's protocol. Sequences of oligonucleotide primers used in PCR amplifications and DNA sequencing for multilocus sequence typing (MLST) were as described previously (Carbone *et al.*, 2007; Moore *et al.*, 2009). DNA sequences for each locus were aligned and manually adjusted using Sequencher Version 4.7 (Gene Codes Corporation, Ann Arbor, MI); alignments were exported as NEXUS files and imported into SNAP Workbench (Price, Carbone, 2005). Multiple sequence alignments for each locus were collapsed separately into haplotypes using SNAP Map (Aylor *et al.*, 2006) and combined using SNAP Combine (Aylor *et al.*, 2006) for inference of MLSTs. Collapsing into haplotypes was performed with the options of recoding insertions/deletions (indels) for maximal MLST resolution.

MLSTs

Genome-wide recombination events arising from independent assortment of chromosomes were detected by examining MLSTs based on variation at eight loci that span four

chromosomes: *aflC*, *aflG/aflL*, *aflV/aflW*, *aflW/aflX* and *mfs* located on chromosome 3; the microsatellite locus AF17 (Grubisha, Cotty, 2009b) on chromosome 2; and the mating type (*MAT*) and tryptophan synthase (*trpC*) genes located on chromosomes 6 and 4, respectively. Missing AF genes were scored as null alleles. For each locus, we tested the null hypothesis of no significant difference in the frequency of parental alleles in the progeny sampled from each cross using a binomial test implemented in MS Excel. In crosses that produce progeny with no crossovers in the AF cluster, we would expect Mendelian segregation of loci on different chromosomes to result in only $(0.5)^4$ or 6% of the progeny sharing a parental MLST.

We examined a cluster-specific MLST for reconstructing crossover events in the AF gene cluster. This was based on a portion of the polyketide synthase gene (*aflC*) that contains a nonsense mutation rendering AF36 non-aflatoxigenic (Ehrlich, Cotty, 2004), three intergenic regions in the AF cluster that span the recombination hotspot (*aflG/aflL*, *aflV/aflW*, and *aflW/aflX*) (Moore *et al.*, 2009), and a gene belonging to the major facilitator superfamily (*mfs*), which is tightly linked to the AF cluster and is present in strains with full, partial or completely missing cluster genes (Moore *et al.*, 2009). A crossover between these loci in the parents would result in progeny genotypes with a different combination of parental alleles.

Array comparative genome hybridization (aCGH)

Representative progeny showing recombinant AF gene clusters, as determined by cluster-specific MLSTs, along with their respective parental strains were further subjected to aCGH to corroborate inferences from cluster-based MLSTs and to possibly identify additional

crossovers within the cluster. Previous work has shown that aCGH provides sufficient resolution to examine intraspecific genetic variation and strain-specific variation in gene content in *A. flavus* (Fedorova *et al.*, 2009). aCGH of parent-offspring trios (two parents plus one progeny) was based on hybridization of total genomic DNA to whole genome *A. flavus* Affymetrix GeneChip microarrays. Each array comprises 12,834 predicted genes and 397 predicted antisense transcripts of *A. flavus* NRRL 3357 (Payne *et al.*, 2008). Each gene is represented by twelve 25-mer-oligonucleotide probes located at the 3' end of the gene. The array includes tiling with partially overlapping probes in the intergenic regions of the AF biosynthetic pathway from *aflF* to *nadA*. Genomic DNA was labeled using the BioPrime DNA labeling System (Invitrogen Catalogue No. 18094-011) as follows: (1) fungal DNA in the amount of 300-350 ng was mixed with 60 μ l of 2.5X random primers (BioPrime kit) and 132 μ l of dH₂O; (2) the reaction mixture was denatured in an Eppendorf thermocycler at 99 °C for 10 min and then cooled at 4 °C for 15 min; (3) 15 μ l of 10X dNTPs containing biotin dCTP and 3 μ l Klenow polymerase were added and incubated at 25 °C in a thermocycler for 18 h; (4) the reaction mixture was precipitated by adding 15 μ l of 3M NaOAc and 400 μ l of cold 95% ethanol, followed by incubation at 20 °C for 15 min; (5) samples were centrifuged at high speed in a refrigerated microcentrifuge for 20 min; and (6) the pellets were washed with 500 μ l ice-cold 70% ethanol, centrifuged and then vacuum dried with heating for 10 min. DNA was resuspended in 100 μ l of dH₂O, and 5 μ l was used to check the quality of the labeled DNA on a gel.

Each sample was analyzed by the microarray laboratories of Expression Analysis (Raleigh-Durham NC). Briefly, 300-350 ng of fragmented genomic DNA was diluted in

hybridization buffer (MES, NaCl, EDTA, Tween 20, Herring Sperm DNA, Acetylated BSA) containing biotin-labeled OligoB2 and Eukaryotic Hybridization Controls (Affymetrix). The hybridization cocktail was denatured at 99 °C for 5 minutes, incubated at 45 °C for 5 minutes and then injected into a GeneChip cartridge. The GeneChip array was incubated at 42 °C for at least 16 hours in a rotating oven at 60 rpm. GeneChips were washed with a series of nonstringent (25 °C) and stringent (50 °C) solutions containing variable amounts of MES, Tween20 and SSPE. The microarrays were then stained with streptavidin-phycoerythrin, and the fluorescent signal was amplified using a biotinylated antibody solution. Fluorescent images were detected in a GeneChip® Scanner 3000 and array data were extracted using the GeneChip Operating System v 1.1 (Affymetrix). Array images were further inspected for defects or debris. Quality control was based on a comparison of the conformity of hybridization controls, scaling factor, and noise; percent detection metrics were analyzed to determine if any outliers were present.

Parent-offspring trio heat maps

Array data for all strains examined using aCGH were imported into JMP genomics (SAS, Cary, NC, USA) for further transformation and normalization. Data were log₂ transformed and normalized using Loess normalization. Because we are interested in detecting differences in hybridization intensities, no background correction was applied. The normalized and transformed data were exported in MS Excel format. All probe sets for genes between the telomere on the right arm of chromosome 3 (abbreviated 3R) up to the CPA cluster, for the adjacent AF cluster genes and intergenic regions, and for the flanking

sugar cluster genes were selected and ordered by their physical location on chromosome 3R (Table S2.1). A heat map and corresponding color scale for this telomeric/subtelomeric region was automatically generated for each of nine parent-offspring trios using the `imagesc` function in Matlab (MathWorks Inc., Natick, MA). For each trio, we evaluated parent/offspring transmission by comparing hybridization intensities from the arrays. We used a simple squared difference between measurements and aggregated the values within a window by summing across probes. In the analyses, p_j^1 , p_j^2 and o_j denote the normalized probe j intensities for parent 1, parent 2 and their offspring, respectively. Crucially, at polymorphic sites we expect the smaller of $(p_j^1 - o_j)^2$ and $(p_j^2 - o_j)^2$ to associate with inheritance and also expect a switch of parental origin at recombination breakpoints. To assess the proximity of probe j to a breakpoint, we sum over neighboring probes

$\sum_{i=1}^{100} (p_{j-i}^1 - o_{j-i})^2 + (p_{j+i}^2 - o_{j+i})^2$ to aggregate signal within a 201-probe window centered around the probe of interest. This value is divided by $\sum_{i=-100}^{100} (p_{j+i}^1 - o_{j+i})^2 + (p_{j+i}^2 - o_{j+i})^2$ to obtain a test statistic t bounded between 0 and 1, with values close to either extreme indicative of a recombination breakpoint.

Genome-wide parentage plots

We examined genome-wide aCGH data for evidence of independent assortment and crossing over in the nine parent-offspring trios. *A. flavus* probe data were normalized using Loess normalization but not log2 transformed. We calculated the median of the $(p_j^2 - o_j)^2$ in a

five-probe window minus the median of the $(p_j^1 - o_j)^2$ in the same five-probe window. This statistic measures the similarity of the offspring to each parent in a five-probe window and was plotted for visual display of putative recombination events. Each red dot in the plots represents one probe (see Fig. S2.1), but the plotted value is based on the five-probe window. The plots for each parent-offspring trio show the degree of similarity of the offspring to each parent on the y-axis and the approximate physical location of each probe on the chromosome. For example, a positive difference translates to an offspring showing more similarity to the top parent in the plot while a negative difference suggests that the offspring is more similar to the bottom parent. Further confirmation of parent-offspring relationships was obtained by mapping MLST loci that distinguish parents and offspring directly on the parentage plot.

Linkage disequilibrium (LD) and recombination analysis

The tiling of intergenic regions on the array allowed for high resolution of recombination breakpoints in the AF gene cluster of F1 progeny. Further resolution of patterns and rates of mutation and recombination in the AF cluster was based on DNA sequence variation in five linked loci (*aflC*, *aflG/aflL*, *aflV/aflW*, *aflW/aflX* and *mfs*). Recombination analyses were based on the experimental population sample, which included parental and F1 progeny strains. We compared estimates of experimental recombination parameters to estimates from a population of *A. flavus* in a peanut field in Herod, Georgia (Horn, Greene, 1995; Moore *et al.*, 2009), which is the source population of six parental strains (IC244, IC278, IC301, IC308, IC277, IC307) used in our experimental crosses; the other five parental strains not used in the population estimates were sampled from Texas (IC313), North Carolina (IC310)

and Alabama (IC311) along a US transect (Horn, Dorner, 1998), from Terrell County in Georgia (NRRL 21882) (EPA, 2004) and from Arizona (AF36) (EPA, 2003). Our hypothesis is that a combined analysis of parents and recombinants arising from crossovers in the AF cluster will accurately reconstruct the recombination block structure observed in nature. This would be supportive evidence that a single round of sexual reproduction could create the patterns of LD and cluster deletions observed in nature. Previously we showed that LD blocks in the AF cluster of *A. flavus* (Moore *et al.*, 2009) coincide with specific cluster deletion patterns of Chang *et al.* (2005).

Recombination analysis in the AF gene cluster was based on variation in the *aflC*, *aflG/aflL*, *aflV/aflW*, *aflW/aflX*, and *mfs* regions, and was performed using five approaches: (1) LD analyses to identify recombination blocks in the cluster using SNAP Clade and Matrix (Bowden *et al.*, 2008), as well as genome-wide analyses including *trpC*, *MAT*, and AF17 to determine the extent of LD between unlinked loci; (2) estimation of the minimum number of recombination events (R_h) using the program RecMin (Myers, Griffiths, 2003); (3) estimation of the population recombination rate per base pair using Hey and Wakeley's γ estimator (Hey, Wakeley, 1997) as implemented in the SITES program; (4) computation of a lower bound on the minimum number of crossovers using HapBound-GC (Song *et al.*, 2007); and (5) reconstruction of a minimal ancestral recombination graph (ARG) using the branch and bound algorithm implemented in the Beagle program (Lyngsø *et al.*, 2005) (<http://www.stats.ox.ac.uk/~lyngsoe/section26/>). All recombination analyses were based on DNA sequence variation that was compatible with an infinite sites mutation model; recoded indels violating this model were excluded using SNAP Map.

MLST, VCG and mycotoxin associations

In a previous study, 79 isolates of *A. flavus* were sampled from a single peanut field (Herod, Georgia, USA) and grouped into 44 VCGs based on vegetative compatibility testing (Horn, Greene, 1995). To determine whether MLSTs inferred for our experimental population are similar to VCGs from nature, we examined variation in seven out of the eight MLST loci (*aflG/aflL*, *aflV/aflW*, *aflW/aflX*, *mfs*, *trpC*, *MAT*, and AF17), which were previously sequenced for all isolates in the Georgia field population (Moore, 2010; Moore *et al.*, 2009). Haplotype diversity was calculated as the weighted average of the estimated diversities in the F1 and natural subpopulations using the H_S statistic described by Hudson *et al.* (1992). Estimates of recombination rates in F1 and natural populations were based on variation in *aflG/aflL*, *aflV/aflW*, *aflW/aflX* and *mfs* regions using the SITES program. Previous work has shown a tight correlation between VCG and AF/CPA concentrations in *A. flavus* (Horn *et al.*, 1996), which suggests that molecular markers that distinguish VCGs can also be useful indicators of mycotoxin diversity. Within each cross we first tested for a significant difference in mean AF and CPA concentrations between progeny and parental isolates using ANOVA followed by a comparison of means using Tukey's multiple comparisons test implemented in the R statistical software (R Development Core Team, 2010). All tests were based on three biological replicates for each isolate except for IC1742, which had two replicates. Significant differences were interpreted within the context of MLSTs based on the five cluster loci (*aflC*, *aflG/aflL*, *aflV/aflW*, *aflW/aflX* and *mfs*) and the eight genome-wide loci (*aflC*, *aflG/aflL*, *aflV/aflW*, *aflW/aflX*, *mfs*, *MAT*, *trpC*, and AF17). Because genome MLST associates with VCG, we examined whether progeny strains that shared a

multilocus haplotype had a significant difference in AF and CPA production.

Results

Sexual crosses

Crosses showed quantitative variation in (1) the number of sclerotia/stromata per slant; (2) the proportion of sclerotia/stromata with ascocarps; and (3) the proportion of sclerotia/stromata containing ascospore-bearing ascocarps (Table 2.1). Four strains (IC244, IC277, IC311, and NRRL 21882) that were self-crossed produced abundant sclerotia and harbored sterile ascocarps as reported in *A. nomius* (Horn *et al.*, 2011); the percentage of sclerotia with sterile ascocarps was between 0.3 (NRRL 21882) and 14.0 (IC311). In crosses between strains of compatible mating type, the percentage of sclerotia/stromata with ascospore-bearing ascocarps ranged from 3.8 to 79.2. The number of sclerotia produced did not correlate with fertility (formation of ascospore-bearing ascocarps), as seen in cross IC278 × AF36, which had the highest fertility but was one of the lowest sclerotial producers (Table 1). As reported previously in *A. parasiticus* (Horn *et al.*, 2009b) and *A. flavus* (Horn *et al.*, 2009a), the same parental strain in different crosses yielded different fertilities; for example, AF36 had the highest fertility when crossed with IC278 and the lowest fertility when crossed to IC301 (Table 2.1).

Mycotoxin heritability

Mean parental and progeny AF B₁, B₂ and CPA concentrations (µg/mL; *n* = 3) are shown in Table 2. The calculated heritability (h^2) was 0.72 ± 0.24 (95% CI: [0.16, 1.28]) for AFs

B_1+B_2 , and 0.87 ± 0.20 (95% CI: [0.39, 1.35]) for CPA production (Fig. 2.1); the remaining proportion of the variance for each can be explained by genotype by environment effects.

MLSTs

As confirmed by genotyping, 82.1% (87/106) of the progeny were recombinants via independent assortment, whereas 5.7% (6/106) of the progeny (IC1650, IC1751, IC2171, IC2205, IC2207, and IC2209) were recombinants in the AF gene cluster (Table 2.2). Cluster loci that flank the recombination hotspot on chromosome 3R (Moore *et al.*, 2009) segregated in a Mendelian fashion such that all of the AF cluster crossovers also showed independent assortment. For example, in cross IC278 \times AF36, genotyping (Table 2) and aCGH (see Fig. S2.1A) show that progeny isolate IC1650 has the genetic background of the AF- parent (AF36) for chromosomes 6, 7 and 8 and inherited chromosomes 2, 4 and 5 of the AF+ parent (IC278). Putative crossovers in these chromosomes are localized to subtelomeric/telomeric regions, giving rise to crossover hybrids as observed in the right arm of chromosome 3 (Fig. S2.1A). Genotyping of cluster loci in IC1650 shows the existence of an AF+ recombinant cluster haplotype H3, with a single crossover in the *aflL* gene region between AF36 and IC278.

There was no significant difference in the frequency of *MATI-1* (48.6%) and *MATI-2* (51.4%) in the progeny pooled from all crosses ($n = 107$, $P = 0.8468$), as is expected for any two segregating loci that are able to assort independently during meiosis. In testing for significance, one offspring (IC1722) had both *MATI-1* and *MATI-2* and was counted twice. Similarly, we examined segregation of linked AF cluster genes (*aflG/aflL*, *aflV/aflW*,

Figure 2.1. A graphical representation of the heritability of aflatoxin (AF) and cyclopiazonic acid (CPA) based on nine *A. flavus* crosses. Each datum point, shown in color, represents one of the nine crosses and was obtained by calculating the midpoint toxin concentrations of both parents (midparent), shown on the x-axis, and the average concentrations of a representative set of 11-12 offspring for each respective cross, shown on the y-axis. The slope of the regression line corresponds to the heritability of the toxin phenotype. For AF, the 95% confidence interval = [0.164681, 1.277303], standard error = 0.235264 and $r^2 = 0.57296$. For CPA, the 95% confidence interval = [0.393246, 1.352344], standard error = 0.202801 and $r^2 = 0.72572$.

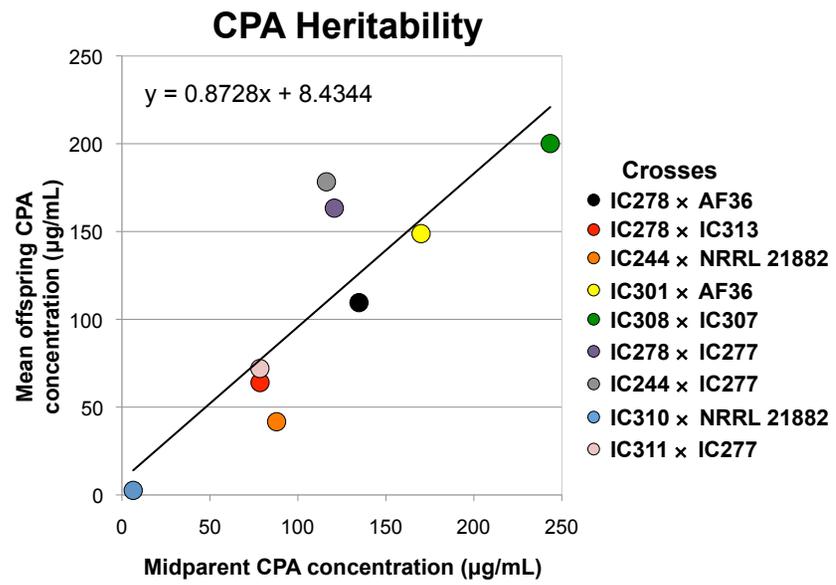
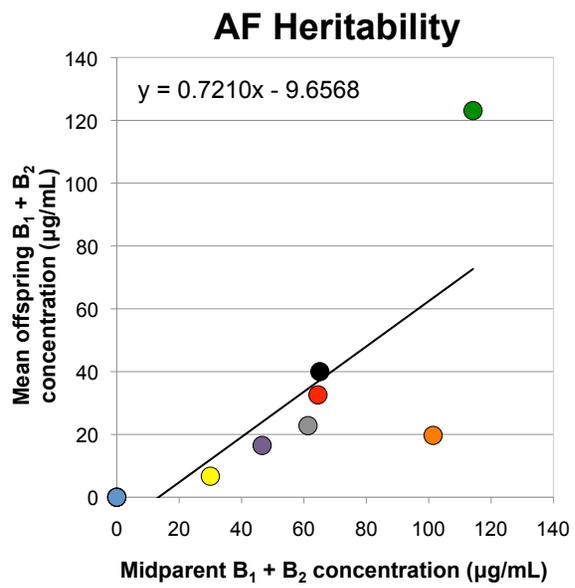


Table 2.2. Multilocus genotypes and toxin phenotypes from nine *Aspergillus flavus* crosses

IC Strain	NRRL Strain ³	Loci examined ¹								Aflatoxin ²				Cluster MLST ⁶	Genome MLST ⁷
		Chr. 6 <i>MAT</i>	Chr. 3 <i>qf/C</i>	Chr. 3 <i>qf/Ci/qf/L</i>	Chr. 3 <i>qf/IV/qf/W</i>	Chr. 3 <i>qf/W/qf/X</i>	Chr. 3 <i>mfs</i>	Chr. 4 <i>trpC</i>	Chr. 2 <i>AF17</i>	B ₁ ($\mu\text{g/mL}$)	B ₂ ($\mu\text{g/mL}$)	Total B ₁ + B ₂ ($\mu\text{g/mL}$) ⁴	CPA ($\mu\text{g/mL}$) ^{4,5}		
AF+ isolates within group IC															
Parents															
IC308	29537	1	H1	H1	H1	H1	H1	H1	H1	18.9 (11.5)	0.4 (0.2)	19.3 (11.7)	279.5 (18.5)	H1	H1
IC307	29536	2	H2	H2	H2	H2	H2	H2	H2	203.2 (24.5)	5.9 (0.5)	209.1 (24.8)	207.3 (15.0)	H2	H2
Progeny															
Ascocarp 2 ⁸															
IC1740	62379	2	H1	H1	H1	H1	H1	H2	H1	85.3 (22.6)	3.2 (0.8)	88.4 (23.4)**	308.6 (31.8)**	H1	H7
IC1741	62380	2	H1	H1	H1	H1	H1	H2	H1	71.5 (7.9)	2.2 (0.2)	73.8 (8.1)**	238.9 (17.2)	H1	H7
IC1742	62381	2	H2	H2	H2	H2	H2	H1	H1	218.4 (33.0)	8.6 (0.9)	227.0 (33.9)*	220.9 (11.8)	H2	H6
IC1743	62382	1	H2	H2	H2	H2	H2	H2	H2	83.7 (12.1)	1.9 (0.2)	85.6 (12.3)**	153.9 (10.4)*	H2	H4
IC1744	62383	1	H2	H2	H2	H2	H2	H2	H1	160.3 (25.8)	3.8 (0.6)	164.1 (26.4)*	172.8 (25.5)*	H2	H5
IC1745	62384	2	H1	H1	H1	H1	H1	H2	H2	154.6 (2.2)	4.5 (0.4)	159.1 (2.5)*	203.2 (49.3)*	H1	H9
IC1746	62385	2	H2	H2	H2	H2	H2	H1	H1	147.0 (34.9)	4.6 (1.1)	151.6 (36.0)*	205.6 (25.3)*	H2	H6
IC1747	62386	2	H1	H1	H1	H1	H1	H2	H1	64.9 (21.0)	2.1 (0.7)	67.0 (21.7)**	164.5 (3.4)	H1	H7
IC1748	62387	1	H2	H2	H2	H2	H2	H2	H2	103.1 (48.0)	2.5 (1.9)	105.6 (49.9)***	163.4 (11.9)*	H2	H4
IC1749	62388	1	H1	H1	H1	H1	H1	H2	H2	62.0 (10.8)	1.6 (0.3)	63.6 (11.0)**	161.7 (17.2)*	H1	H10
IC1750	62389	2	H1	H1	H1	H1	H1	H1	H2	108.4 (31.9)	2.9 (0.9)	111.2 (32.7)***	228.0 (20.3)	H1	H8
IC1751	62390	2	H2	H2	H1	H1	H1	H2	H2	173.9 (9.2)	6.1 (0.6)	180.0 (9.7)*	179.2 (6.5)*	H3	H3
AF+ and AF- isolates within group IC															
Parents															
IC278	29507	1	H1	H1	H1	H1	H1	H1	H1	127.0 (20.2)	3.2 (0.7)	130.2 (20.8)	157.6 (6.1)	H1	H1
AF36 ⁹	18543	2	H2	H2	H2	H1	H1	H2	H2	0 (0)	0 (0)	0 (0)	111.8 (6.8)	H2	H2
Progeny															
Ascocarp 1															
IC1644	62309	1	H1	H1	H1	H1	H1	H1	H1	27.4 (6.3)	0.5 (0.2)	27.8 (6.5)*	166.4 (38.9)**	H1	H1
IC1645	62310	2	H1	H1	H1	H1	H1	H1	H1	78.6 (28.0)	1.7 (0.7)	80.4 (28.7)***	154.0 (8.6)	H1	H7
IC1646	62311	1	H2	H2	H2	H1	H1	H1	H2	0 (0)	0 (0)	0 (0)*	52.2 (21.5)***	H2	H3
IC1647	62312	1	H2	H2	H2	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)*	67.8 (17.9)*	H2	H5
IC1648	62313	1	H1	H1	H1	H1	H1	H1	H2	107.1 (23.4)	3.1 (0.7)	110.2 (24.1)**	146.6 (16.1)	H1	H9
IC1649	62314	2	H1	H1	H1	H1	H1	H2	H1	52.3 (9.3)	1.0 (0.2)	53.3 (9.5)***	116.0 (11.3)	H1	H8
Ascocarp 2															
IC1650	62315	2	H1	H1	H2	H1	H1	H1	H1	85.1 (11.2)	1.7 (0.3)	86.8 (11.6)***	129.8 (4.7)	H3	H6
IC1651	62316	1	H2	H2	H2	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)*	126.1 (9.2)	H2	H5
IC1652	62317	1	H2	H2	H2	H1	H1	H2	H2	0 (0)	0 (0)	0 (0)*	113.0 (14.5)	H2	H4
IC1653	62318	2	H1	H1	H1	H1	H1	H2	H1	48.8 (11.0)	1.0 (0.2)	49.7 (11.2)***	85.0 (7.9)*	H1	H8
IC1654	62319	1	H2	H2	H2	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)*	43.8 (4.8)***	H2	H5
IC1655	62320	2	H1	H1	H1	H1	H1	H1	H1	70.0 (10.5)	1.5 (0.2)	71.5 (10.7)***	112.9 (4.1)	H1	H7
Parents															
IC301	29530	1	H1	H1	H1	H1	H1	H1	H1	59.3 (28.5)	0.8 (0.4)	60.1 (28.9)	222.7 (19.7)	H1	H1
AF36	18543	2	H2	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)	117.3 (8.1)	H2	H2
Progeny															
Ascocarp 1															
IC2165	62368	2	H2	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)*	83.9 (11.7)*	H2	H2
IC2166	62369	1	H1	H1	H1	H1	H1	H2	H1	13.6 (1.9)	0.1 (0.02)	13.7 (1.9)*	209.2 (17.1)	H1	H8
IC2167	62370	2	H1	H1	H1	H1	H1	H1	H1	8.0 (8.6)	0.1 (0.1)	8.1 (8.7)*	179.9 (63.5)	H1	H6
IC2168	62371	2	H2	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)*	167.6 (15.1)	H2	H2

Table 2.2. Continued

IC2169	62372	2	H1	H1	H1	H1	H1	H2	H1	11.4 (2.8)	0.1 (0.05)	11.6 (2.8)*	240.1 (22.01)	H1	H7
IC2170	62373	2	H1	H1	H1	H1	H1	H1	H1	12.6 (2.5)	0.1 (0.03)	12.7 (2.5)*	157.3 (93.3)	H1	H6
IC2171	62374	2	H2	H1	H1	H1	H1	H2	H1	0 (0)	0 (0)	0 (0)*	99.9 (19.4)	H3	H3
IC2172	62375	2	H2	H2	H2	H2	H2	H1	H2	0 (0)	0 (0)	0 (0)*	86.5 (5.0)*	H2	H4
IC2173	62376	2	H1	H1	H1	H1	H1	H1	H1	9.4 (12.9)	0.1 (0.1)	9.5 (13.0)*	157.0 (89.6)	H1	H6
IC2174	62377	2	H2	H2	H2	H2	H2	H2	H1	0 (0)	0 (0)	0 (0)*	100.6 (8.1)	H2	H5
IC2176	62378	1	H1	H1	H1	H1	H1	H2	H1	18.0 (2.9)	0.1 (0.03)	18.1 (2.9)*	154.6 (63.2)	H1	H8
AF+ from group IC, and AF- isolates from group IB															
Parents															
IC244	29473	1	H1	H1	H1	H1	H1	H1	H1	118.5 (31.1)	4.1 (1.4)	122.6 (32.5)	119.0 (4.9)	H1	H1
IC277	29506	2	H2	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)	113.5 (11.1)	H2	H2
Progeny															
Ascocarp 1															
IC2201	62403	2	H1	H1	H1	H1	H1	H2	H1	11.9 (4.1)	0.2 (0.1)	12.1 (4.2)*	62.8 (1.6)	H1	H9
IC2202	62404	1	H1	H1	H1	H1	H1	H1	H1	73.3 (38.7)	2.7 (1.5)	76.0 (40.2)**	152.6 (53.3)	H1	H1
IC2203	62405	1	H1	H1	H1	H1	H1	H2	H2	91.8 (23.0)	2.5 (0.8)	94.4 (23.8)**	146.1 (27.5)	H1	H10
IC2204	62406	1	H2	H2	H2	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)*	525.0 (292.0)***	H2	H4
IC2205	62407	2	H1	H1	H2	H2	H2	H1	H2	58.0 (17.6)	0.8 (0.3)	58.8 (17.9)***	104.5 (23.4)	H4	H8
IC2206	62408	2	H1	H1	H1	H1	H1	H2	H1	31.2 (16.3)	0.6 (0.4)	31.8 (16.7)*	91.2 (12.4)	H1	H9
IC2207	62409	1	H2	H2	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)*	224.5 (7.4)	H3	H3
IC2208	62410	2	H2	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)*	205.8 (10.4)	H2	H2
IC2209	62411	2	<i>H2</i> ¹⁰	<i>H2</i>	<i>H2</i>	<i>H2</i>	<i>H2</i>	<i>H2</i>	<i>H2</i>	0 (0)	0 (0)	0 (0)*	0 (0)	H5	H11
IC2210	62412	2	H2	H2	H2	H2	H2	H2	H1	0 (0)	0 (0)	0 (0)*	118.1 (9.6)	H2	H7
IC2211	62413	2	H2	H2	H2	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)*	240.9 (40.4)	H2	H6
IC2212	62414	1	H2	H2	H2	H2	H2	H2	H1	0 (0)	0 (0)	0 (0)*	268.2 (19.0)	H2	H5
Parents															
IC244	29473	1	H1	H1	H1	H1	H1	H1	H1	195.3 (39.4)	7.5 (1.8)	202.8 (41.1)	175.7 (8.0)	H1	H1
IC316	21882 ¹¹	2	- ¹²	-	-	-	H2	H2	H2	0 (0)	0 (0)	0 (0)	0 (0)	H2	H2
Progeny															
Ascocarp 1															
IC1692	62333	2	H1	H1	H1	H1	H1	H1	H1	68.0 (14.6)	1.8 (0.6)	69.9 (15.2)***	136.5 (5.0)***	H1	H6
IC1693	62334	1	<i>H1</i>	<i>H1</i>	-	-	H2	H1	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H3
IC1694	62335	2	H1	H1	H1	H1	H1	H1	H1	58.3 (23.7)	0.8 (0.4)	59.0 (24.1)***	67.2 (8.3)***	H1	H6
IC1695	62336	2	-	-	-	-	H2	H1	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H5
IC1696	62337	1	H1	H1	H1	H1	H1	H2	H1	57.1 (27.4)	1.2 (0.7)	58.3 (28.1)***	108.5 (31.2)***	H1	H8
IC1697	62338	2	<i>H1</i>	<i>H1</i>	-	-	H2	H1	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H5
IC1698	62339	2	-	-	-	-	H2	H1	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H5
IC1699	62340	1	<i>H2</i>	-	-	-	H2	H2	H1	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H4
IC1700	62341	2	H1	H1	H1	H1	H1	H2	H1	28.6 (5.7)	0.6 (0.1)	29.2 (5.8)*	146.2 (25.5)**	H1	H7
IC1701	62342	2	-	-	-	-	H2	H1	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H5
IC1703	62343	1	-	-	-	-	H2	H1	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H3
Parents															
IC278	29507	1	H1	H1	H1	H1	H1	H1	H1	91.1 (21.0)	2.1 (0.6)	93.2 (21.6)	131.3 (5.1)	H1	H1
IC277	29506	2	H2	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)	110.1 (8.9)	H2	H2
Progeny															
Ascocarp 1															
IC2189	62391	1	H2	H2	H2	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)*	82.0 (26.4)	H2	H3
IC2190	62392	2	H2	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)*	122.8 (14.3)	H2	H2
IC2191	62393	1	H1	H1	H1	H1	H1	H2	H2	64.8 (23.4)	1.4 (0.6)	66.2 (24.1)**	109.6 (7.1)	H1	H6
IC2192	62394	1	H2	H2	H2	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)*	160.4 (14.2)	H2	H3
IC2193	62395	1	H2	H2	H2	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)*	579.2 (48.8)***	H2	H3
IC2194	62396	1	H1	H1	H1	H1	H1	H1	H1	42.4 (6.6)	0.6 (0.2)	42.9 (6.8)***	99.3 (9.2)	H1	H1

Table 2.2. Continued

IC2195	62397	1	H1	H1	H1	H1	H1	H2	H1	30.4 (9.3)	0.4 (0.1)	30.8 (9.4)***	84.2 (8.2)	H1	H7
IC2196	62398	2	H2	H2	H2	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)*	164.1 (16.9)	H2	H5
IC2197	62399	1	H2	H2	H2	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)*	118.4 (20.1)	H2	H3
IC2198	62400	1	H1	H1	H1	H1	H1	H1	H2	17.0 (3.4)	0.3 (0.1)	17.3 (3.4)*	95.9 (11.0)	H1	H8
IC2199	62401	1	H1	40.2 (3.6)	0.6 (0.1)	40.8 (3.6)***	177.0 (7.5)**	H1	H1						
IC2200	62402	1	H2	0 (0)	0 (0)	0 (0)*	166.3 (41.7)	H2	H4						
Parents															
IC278	29507	1	H1	125.7 (48.9)	3.3 (1.6)	129.1 (50.4)	156.9 (55.4)	H1	H1						
IC313	35739	2	–	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)	0 (0)	H2	H2
Progeny															
Ascocarp 1															
IC1668	62321	2	–	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H2
IC1669	62322	2	H1	H1	H1	H1	H1	H2	H2	46.9 (17.4)	0.8 (0.5)	47.7 (17.8)*	68.8 (21.7)***	H1	H7
IC1670	62323	2	H1	H1	H1	H1	H1	H2	H1	67.9 (16.2)	1.4 (0.3)	69.2 (16.5)***	117.9 (35.6)**	H1	H10
IC1671	62324	2	H1	53.5 (11.0)	1.0 (0.2)	54.5 (11.2)***	88.5 (10.3)***	H1	H8						
IC1672	62325	2	H1	H1	H1	H1	H1	H2	H2	58.2 (12.8)	1.5 (0.4)	59.6 (13.3)***	101.5 (10.2)**	H1	H7
IC1673	62326	2	H1	H1	H1	H1	H1	H2	H1	47.9 (5.1)	0.8 (0.1)	48.7 (5.2)*	109.4 (11.9)**	H1	H10
IC1674	62327	2	–	H2	H2	H2	H2	H2	H1	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H6
IC1675	62328	2	–	H2	H2	H2	H2	H1	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H5
IC1676	62329	1	–	H2	H2	H2	H2	H2	H2	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H3
IC1677	62330	1	H1	H1	H1	H1	H1	H2	H2	37.9 (10.6)	0.7 (0.3)	38.5 (10.9)*	126.1 (21.3)**	H1	H11
IC1678	62331	1	–	H2	H2	H2	H2	H2	H1	0 (0)	0 (0)	0 (0)*	0 (0)*	H2	H4
Ascocarp 2															
IC1679	62332	2	H1	H1	H1	H1	H1	H1	H2	70.9 (20.3)	1.5 (0.5)	72.4 (20.8)***	155.8 (9.2)**	H1	H9
AF- isolates within group IB															
Parents															
IC310	35736	1	H1	0 (0)	0 (0)	0 (0)	12.7 (2.8)	H1	H1						
IC316	21882	2	–	–	–	–	H2	H1	H2	0 (0)	0 (0)	0 (0)	0 (0)	H2	H2
Progeny															
Ascocarp 1															
IC1716	62344	1	H2	H2	–	–	H2	H1	H2	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H3
IC1717	62345	2	–	–	–	–	H2	H1	H2	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H2
IC1718	62346	1	–	–	–	–	H2	H1	H2	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H3
IC1719	62347	1	H1	H1	H1	H1	H1	H1	H2	0 (0)	0 (0)	0 (0)	0 (0)*	H1	H8
IC1720	62348	2	H1	0 (0)	0 (0)	0 (0)	20.9 (1.7)***	H1	H6						
IC1721	62349	1	–	–	–	–	H2	H1	H2	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H3
IC1722	62350	1 & 2	H1	H1	H1	H1	H1	H1	H2	0 (0)	0 (0)	0 (0)	10.2 (2.8)**	H1	H7
IC1723	62351	2	–	–	–	–	H2	H1	H2	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H2
IC1724	62352	1	H2	H3	–	–	H2	H1	H1	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H4
IC1725	62353	2	–	–	–	–	H2	H1	H1	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H5
IC1726	62354	2	–	–	–	–	H2	H1	H1	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H5
IC1727	62355	2	–	–	–	–	H2	H1	H1	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H5
Parents															
IC311	35737	1	–	H1	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)	36.8 (20.8)	H1	H1
IC277	29506	2	H1	H2	H1	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)	120.0 (34.0)	H2	H2
Progeny															
Ascocarp 1															
IC1764	62356	1	–	H1	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)	138.7 (15.2)*	H1	H1
IC1765	62357	1	H1	H2	H1	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)	100.2 (14.7)*	H2	H3
IC1766	62358	1	–	H1	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)	48.5 (1.9)**	H1	H1
Ascocarp 3															
IC1767	62359	1	–	H1	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)	29.2 (5.1)**	H1	H1

Table 2.2. Continued

Ascocarp 8															
IC1768	62360	1	H1	H2	H1	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)	45.5 (16.0)**	H2	H3
IC1769	62361	1	–	H1	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)	45.9 (6.8)**	H1	H1
Ascocarp 9															
IC1770	62362	1	<i>H2</i>	H1	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)	128.9 (9.1)*	H1	H1
IC1771	62363	2	–	H1	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)	47.5 (4.1)**	H1	H5
IC1772	62364	1	H1	H2	H1	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)	144.4 (26.4)*	H2	H3
IC1773	62365	1	H1	H2	H1	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)	3.8 (3.8)**	H2	H3
IC1774	62366	1	H1	H2	H1	H2	H2	H1	H1	0 (0)	0 (0)	0 (0)	84.7 (36.6)	H2	H3
IC1775	62367	1	–	H1	H1	H1	H1	H1	H1	0 (0)	0 (0)	0 (0)	46.3 (15.2)**	H1	H1

¹Parental mating-type (*MAT*) designations for NRRL 29473, 29506, 29507, 29530, 29536, 29537 are from Ramirez-Prado et al. (2008); *aflV/aflW* is approximately 16 kb downstream of *aflG/aflL* on Chr. 3; *aflW/aflX* is approximately 2 kb downstream of *aflV/aflW* on Chr. 3; *mfs* is approximately 14 kb downstream of *aflW/aflX* on Chr. 3 [72]. The *aflG/aflL*, *aflV/aflW*, and *aflW/aflX* loci are intergenic, and the *aflC* locus is within the exon at the location of the nonsense mutation in AF36. Each cross is separated by gray blocks in which the parents are indicated.

²Aflatoxin values are means of three replications; standard deviations are in parentheses.

³Strain numbers (NRRL) from Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

⁴Significant ($P \leq 0.05$) differences in total aflatoxin and CPA concentrations are indicated between a progeny isolate and the *MAT1-1* parent (*), the *MAT1-2* parent (**) or both (***)

⁵Cyclopiazonic acid values are means of 3 replications; standard deviations are in parentheses.

⁶Cluster MLST refers to the collapsing of genetic data comprising the loci within the aflatoxin cluster (chr. 3R), including the linked gene locus *mfs*. The presence of haplotypes that differ from the parents (H1, H2) indicates a crossover recombination event.

⁷Genome MLST incorporates all loci examined into the collapsing of genetic data. The presence of haplotypes that differ from the parents (H1, H2) indicates recombination due to independent assortment and/or crossing over.

⁸For each cross, ascospores from different ascocarps were examined separately.

⁹AF36 = IC1179.

¹⁰Haplotype designations in italics represent putative hidden alleles and were excluded from cluster and genome MLST determinations.

¹¹NRRL 21882 = Afla-Guard® strain.

¹²Signifies a missing locus within the genotype of the particular strain.

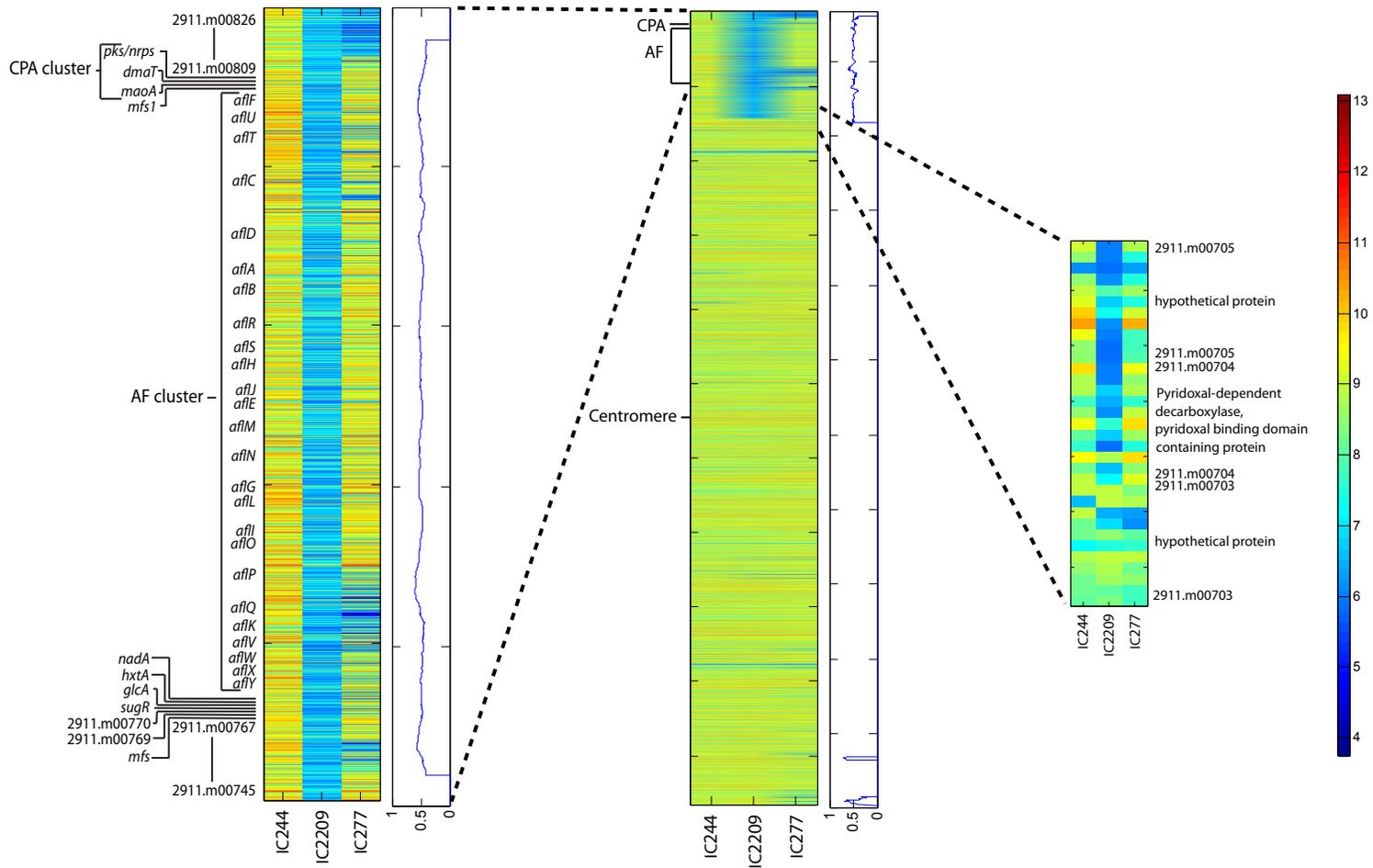
aflW/aflX, *mfs*), *trpC*, *MAT* and AF17 in each cross, and with the exception of *MAT* in crosses IC311 × IC277 and IC278 × IC277 where *MAT1-1* predominates, there were no significant differences in the frequency of the two parental alleles in the progeny (data not shown). All sequence data were submitted to GenBank under Accession numbers JF418181 – JF418920.

MLST and cryptic heterokaryons

While the majority of F1 ascospores were homokaryotic, the existence of both parental alleles for AF cluster genes in some progeny suggests the existence of genetically unbalanced nuclei, where one nucleus is dominant and determines the AF and CPA phenotype. For example, in cross IC244 × NRRL 21882, progeny strains IC1693 and IC1697 were nontoxicogenic and were found to be identical to NRRL 21882 at the *mfs* locus; however, we were also able to amplify and sequence the *aflC* and *aflG/aflL* loci that matched IC244, the toxicogenic parental strain. Moreover, in cross IC310 × NRRL 21882 at least one progeny strain, IC1722, was heterokaryotic for mating type (Table 2.2). Differences in intensities of ethidium-bromide-stained *aflC* and *aflG/aflL* amplicons compared to *mfs* after gel electrophoresis (data not shown) suggest that there may be variation in nuclear copy number, which was also observed using aCGH; quantitative PCR will be done to validate these findings. A specific case in point is strain IC2209 from cross IC244 × IC277. According to the aCGH, strain IC2209 has an approximately 374-kb deletion of the telomeric/subtelomeric end of chromosome 3R (Fig. 2.2; Table S2.1). This evidence of segmental aneuploidy in

Figure 2.2. Heat maps of chromosome 3 based on array comparative genome hybridization (aCGH) of progeny strain IC2209 and parental strains IC244 and IC277. In each heat map trio, IC2209 is shown in the center column and IC244 and IC277 are shown in the adjacent columns. In the center is a graphical representation of the aCGH data for chromosome 3 showing the approximate location of the centromere and the AF and CPA clusters. The trio map at the left is based on probes spanning predicted genes 2911.m00826 - 2911.m00745 on chromosome 3R in *A. flavus* NRRL 3357. Supplemental Table S1 lists the putative functions of these genes. The genes involved in CPA (*pks-nrps-maoA*) and AF (*aflF-nadA*) biosynthesis are labeled. Within each heat map, red bars indicate high sequence similarity to the reference genome (NRRL 3357), which is the result of strong hybridization of the target DNA to the probes on the array; inversely, blue bars indicate weak hybridization and high sequence dissimilarity, or alternatively, the absence of a gene or DNA segment. For example, the region spanning the AF and CPA clusters and extending into the subtelomeric region of IC2209 matches neither parental heat map. This region of weak hybridization may be the result of a deletion or sequence dissimilarity due to a duplication and translocation of another genomic region, as in mutant strain 649 (Smith *et al.*, 2007). A deletion of this large segment is supported by the CPA-/AF- toxin phenotype of IC2209, which is distinct from the parents that are both CPA+ (Table 2). This approximately 374-kb deletion extends to probes spanning predicted genes 2911.m00704 (pyridoxal-dependent decarboxylase, pyridoxal binding domain containing protein) as shown in the rightmost trio heat map. On the right of the two leftmost trio maps is a plot of test statistic, *t*. See Figure 2.3 legend for a detailed description of *t*.

Chromosome 3



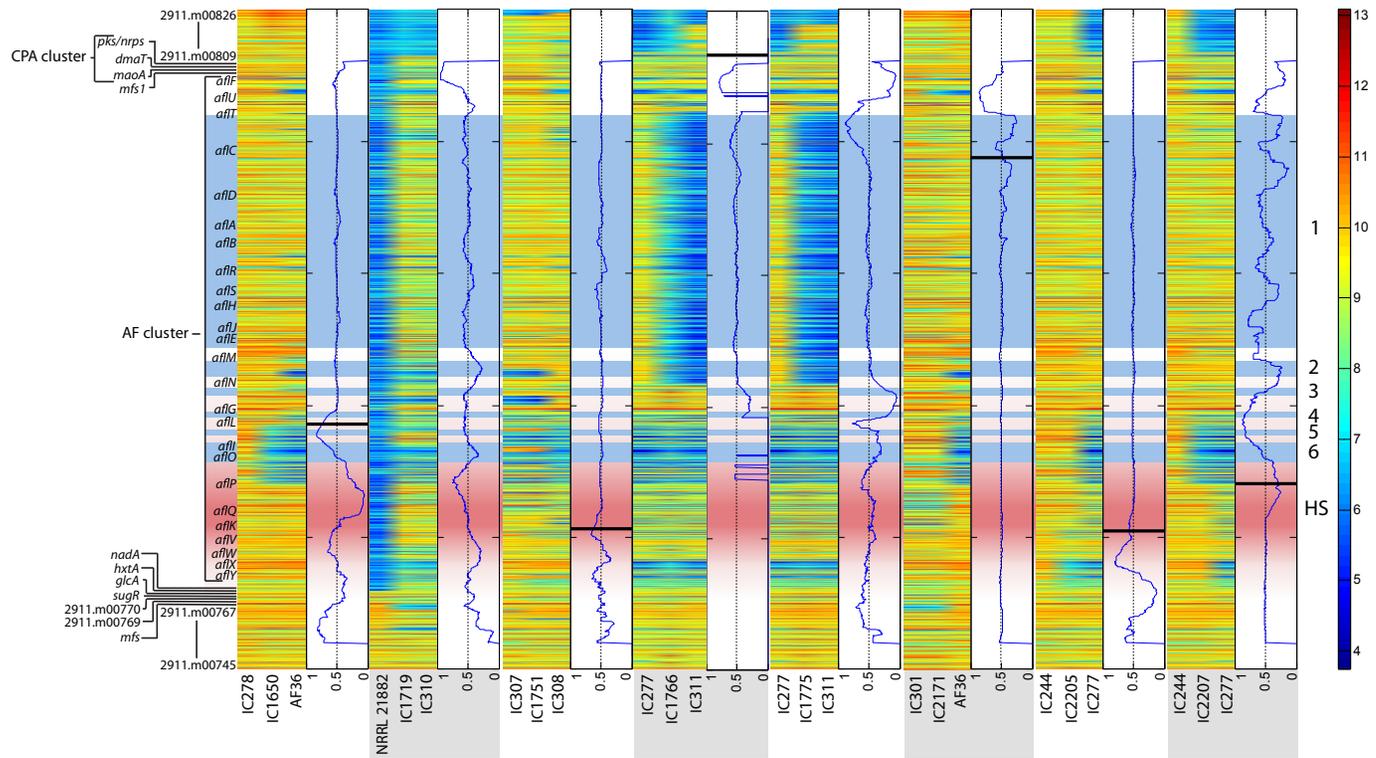
IC2209 is inconsistent with the amplification and sequencing of *aflC*, *aflG/aflL*, *aflV/aflW*, *aflW/aflX*, and *mfs* regions in this strain, which indicates that IC2209 should be identical to parental strain IC277 (Table 2.2); however, unlike IC277, progeny strain IC2209 does not make CPA, which supports the aCGH deletion pattern in chromosome 3R. Since aCGH is based on hybridization of genomic DNA without an amplification step, aCGH is more sensitive to copy number variation and such hybridization will favor the majority nucleus in any given strain.

Non-Mendelian inheritance of non-parental extra-genomic alleles was also observed in some crosses. In this case, the allele was not detected in the parents but was amplified and sequenced in the offspring. For example, progeny strain IC1699 from cross IC244 × NRRL 21882 shared the same *mfs* allele as NRRL 21882, but the *aflC* allele sequenced in IC1699 was not identical to the corresponding parent IC244, but instead matched the *aflC* allele in strain IC310. Even more cryptic alleles were detected in strain IC1724 from cross IC310 × NRRL 21882 with at least two different non-parental alleles: *aflC* was identical to strains IC244, IC278, IC301 and IC307, and *aflG/aflL* was identical to strains IC277 and IC308. Furthermore, strain IC1716 from the same cross had a unique *aflG/aflL* sequence that was not found in the natural population (Tables S2.2 and S2.3). Only the *mfs* allele in the non-cluster region segregated in the progeny in a Mendelian fashion, which suggests that strains may be heterokaryotic only in the telomeric/subtelomeric region of chromosome 3R.

Parent-offspring trio heat maps

Heat maps of eight parent-offspring trios identified putative crossovers in the AF cluster of six progeny and showed that inferred breakpoints in parents based on single feature polymorphisms (SFPs) coincide with recombination blocks and hotspots observed in natural populations according to single nucleotide polymorphisms (SNPs) (Fig. 2.3). Because our cluster-specific genetic markers are missing in strains with partial or completely missing genes, we examined the possibility of crossovers using aCGH for progeny isolates IC1775 and IC1719 derived from parents with partial (IC311) or completely missing clusters (NRRL 21882), respectively. No crossover events were detected in chromosome 3R for the NRRL 21882, IC1719 and IC310 trio (Figs 2.3, S2.1B), as well as for the IC277, IC1775 and IC311 trio (Figs. 2.3, S2.1E), which agreed with genotyping results. The hotspot region from *aflO* to *aflW* showed higher recombinant activity in experimental crosses than the coldspot region spanning *aflT* to *aflE*, which corresponds to recombination block 1. Recombinants IC1751, IC2205 and IC2207 are the result of crossovers within the hotspot, localized at the *aflK/aflV* intergenic region and *aflP*, whereas IC1650 is the result of a crossover in *aflL*, which is the gene separating linkage disequilibrium blocks 4 and 5 (Fig. 2.3). A single crossover was detected in the cold spot region starting with *aflC* (including the nonsense mutation) in cross IC301 × AF36 such that offspring IC2171 inherited both AF- and CPA+ traits from the AF36 parent; IC2171 was otherwise identical to the AF+ parent (IC301) in the AF/sugar clusters and adjacent subtelomeric regions (Fig. 2.3). All aCGH data were submitted to the Gene Expression Omnibus (GEO) under Accession numbers GSM679190 – GSM679207.

Figure 2.3. Heat maps of eight *A. flavus* parent/offspring trios. Each trio map is based on probes spanning predicted genes 2911.m00826 - 2911.m00745 on chromosome 3R in *A. flavus* NRRL 3357 (see Table S1). On the right of each trio map is a plot of the test statistic, t . In general the test statistic is bounded between 0 and 1, with intermediate values indicative of similarity across the trio and values close to either extreme indicative of a putative recombination breakpoint; a horizontal black line indicates the location of a potential crossover breakpoint. For example, offspring strain IC1650 is more similar to parent strain AF36 immediately below *aflL* and more similar to parent strain IC278 immediately above *aflL*. LD blocks reported in Moore *et al.* (2009) are numbered from 1 to 6 and are shown as light blue-shaded regions; similarly, the recombination hotspot (HS) region is shown as a red-shaded gradient region.



Genome-wide parentage plots

Both independent assortment and crossovers were observed in genome-wide aCGH parentage plots for the nine parent-offspring trios (Fig. S2.1). Sequence data from linked loci in the AF cluster (*aflW/aflX* and *mfs*) and unlinked loci comprising the MLST (*AF17*, *MAT*, *trpC*) confirmed that patterns of inheritance observed in parentage plots are the result of SFPs between the labeled target DNA hybridized to unlabeled *A. flavus* NRRL 3357 probes fixed on the array. Known patterns of descent from sequenced MLST loci (position indicated with vertical blue lines in parentage plots) confirm inferred independent assortment and crossover events based on array SFP data. For example, the *trpC* sequence on chromosome 4 in progeny IC1650 is identical to parent IC278, whereas *MAT1-2* on chromosome 6 is from the AF36 parent (Table 2.2, Fig. S2.1A).

In the absence of crossovers, chromosomal inheritance in progeny isolates can be deduced by comparing SFPs in parentage plots (Fig. S2.1). Frequent crossover events resulted in most progeny having chromosomes of mixed parental origin. Crossovers were predominantly located in telomeric/subtelomeric regions. For example, in the AF gene cluster for IC1650, the *aflC* and *aflG/aflL* regions (rightmost two vertical blue lines on chromosome 3 graph in Fig. S2.1A) are from parent IC278, while the *aflV/aflW*, *aflW/aflX* and *mfs* regions (leftmost three vertical lines) are from parent AF36, suggesting one crossover in chromosome 3R; the telomeric end of the left arm of chromosome 3 also shows evidence of a crossover but in this case IC1650 is more similar to AF36 (Fig. S2.1A). In some progeny, crossovers were localized to centromeric regions of chromosomes. For example, IC2205 inherited the right arm of chromosome 8 from the IC277 parent and the left

arm from the IC244 parent. In IC1650 and IC2205, crossovers coincide with the putative location of centromeres on chromosomes 1 and 5, respectively.

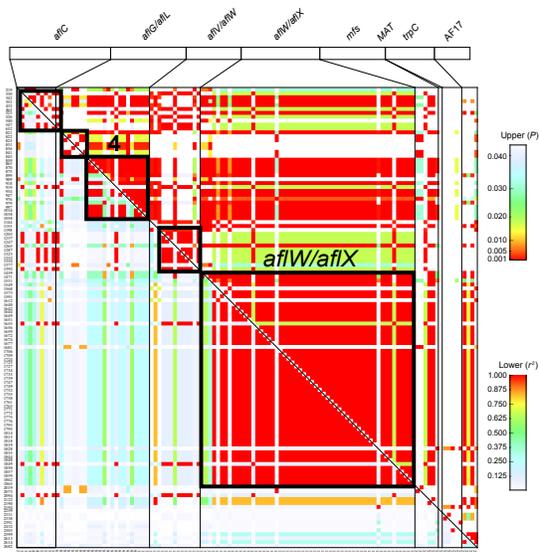
The position of recombination breakpoints was conserved in progeny from different crosses. For example, the crossover in the right arm of chromosome 1 was observed in both progeny IC1650 (parental cross AF36 × IC278; Fig. S2.1A) and IC2207 (parental cross IC244 × IC277; Fig. S2.1H). The *A. flavus* oligonucleotide-based microarrays allow recombination breakpoint resolution of approximately 15 kb based on the spacing of probes between adjacent genes. The higher probe density in the tiled intergenic regions of the AF cluster provides higher breakpoint resolution. The minimal probe coverage and lack of DNA sequence variation in coding regions elsewhere in the genomes made it difficult to estimate an upper bound on the number of recombination events along chromosomes, but overall at least one crossover per chromosome arm was observed when there was sufficient DNA sequence variation to distinguish parents. Breakpoint resolution was particularly poor in crosses where the parents were very similar; for example, IC311 and IC277 are members of lineage IB and share a recent common ancestor in the AF cluster (Moore *et al.*, 2009). In this cross, the parentage of IC1775 is only clearly resolved in the AF cluster on chromosome 3 (Fig. S2.1E) where IC1775 is most similar to IC311, which has a deletion of the cluster from *aflT* to *aflM* (Moore *et al.*, 2009); elsewhere in the genome there is a paucity of SFPs for tracking recombination.

Linkage disequilibrium (LD) and recombination analysis in experimental population

Analysis of DNA sequence variation in cluster loci of experimental populations revealed

Figure 2.4. A graphical representation of linkage disequilibrium (LD) and site compatibility based on variation in eight MLST loci in the *A. flavus* experimental population. The LD and compatibility matrices are based on 118 polymorphisms shown in Table S2.4. At the top of both plots is a schematic of the MLST loci. In the LD plot, the lower triangular matrix represents r^2 , the coefficient of determination between the allelic states at pairs of sites, and the upper triangular matrix shows the significance level between pairs of polymorphic sites (including sites with low allele frequencies) calculated using Fisher's Exact test; colored shading denotes statistical significance and strength of associations. LD analysis reveals five distinct blocks that mirror the block-like pattern of LD previously observed in the natural *A. flavus* population (Moore *et al.*, 2009). LD breaks down rapidly between the non-cluster loci, *MAT*, *trpC* and AF17, as expected for loci that are segregating in a Mendelian fashion. There is significant incompatibility between blocks 4 and *aflW/aflX*, as shown in the compatibility matrix where white and black squares denote pairs of compatible and incompatible sites, respectively. All variation within blocks is fully compatible, indicating shared common ancestry among parents and progeny, as well as descent with recombination as shown in Figure 2.5.

LD of experimental population



Site compatibility of experimental population

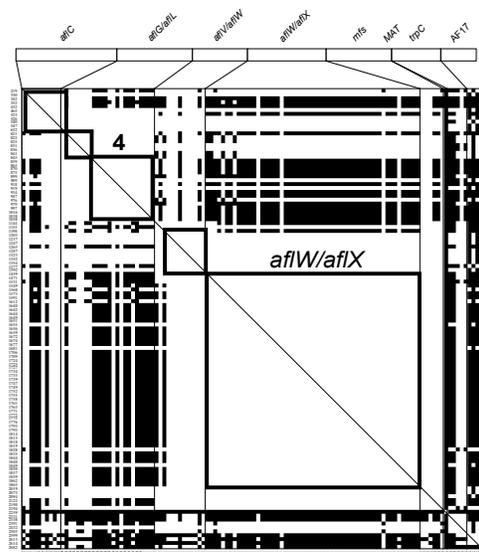


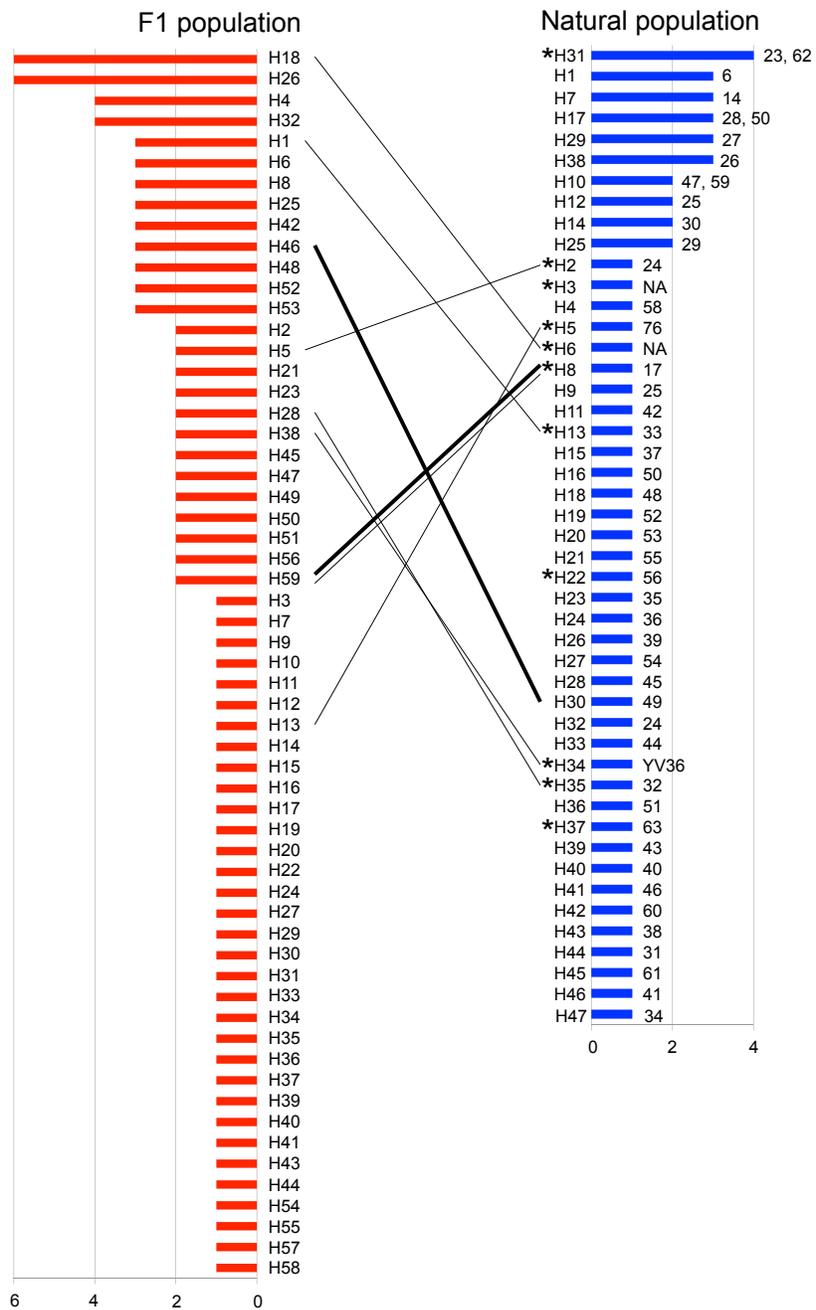
Figure 2.5. One possible most parsimonious reconstruction of the ancestral recombination graph (ARG) inferred for all polymorphisms in *aflC*, *aflG/aflL*, *aflV/aflW*, *aflW/aflX* and *mfs* in the *A. flavus* experimental population. The ARG shows all mutation and recombination paths separating multilocus haplotypes (red ellipses; Table S2.7). Coalescent nodes are shown in green; a yellow dot (the highest point in the ARG) denotes the inferred ancestral (root) sequence. The ARG was rooted with haplotype H1 (IC277). The direction of paths is from the top of the ARGs (past) to the bottom (present); moving backwards in time, one of three events (mutation, coalescence or recombination) is possible. The paths leading to the recombination nodes (blue ellipses) are labeled with a P (prefix) or S (suffix), indicating the 5' and 3' segments of the recombinant sequence, respectively; the number labels on the paths indicate the number of polymorphisms. The numbers in the blue ellipses indicate the variable position immediately to the left of the recombination breakpoint (Table S2.6). The thickened lines in the ARG show the most parsimonious path from the parental isolates IC277 (H1) and IC244 (H5) to the recombinant progeny isolate IC2207 (H12).

significant LD ($r^2 > 0.8$; $P < 0.01$) among contiguous pairs of polymorphic sites, resolving five distinct recombination blocks (Fig. 2.4; Tables S2.4 and S2.5), two of which were described previously from the natural population from which six of the eleven parental isolates in this study originated (Moore *et al.*, 2009). The recovery of the natural block structure in experimental crosses further suggests that crossovers in the progeny coincide with recombination hotspots in parental strains, which was also observed in parent-offspring trio heat maps (Fig. 2.3). Estimates of the minimum number of recombination events and overall recombination rate per base pair in the cluster for parents and F1 progeny were 10 and 0.002458, respectively. The ARG shows one most parsimonious reconstruction of mutation and recombination events in the ancestral history of the experimental population (Fig. 2.5; Tables S2.6 and S2.7). The ARG portrays known patterns of descent for all parents and progeny. For example, haplotypes H1 with parental strain IC277 and H5 with parental strain IC244 (Table S2.7) give rise to recombinant haplotype H12 (IC2207), which is shown with bold lines in Fig. 2.5. In some cases the ARG provides evidence of recombination in the parents. For example, AF- haplotype H2 (includes AF36) shows a previous history of recombination, with AF+ haplotype H9 (IC1650) and AF- haplotype H3 (IC2171) as the immediate parents.

MLST, VCG and mycotoxin associations

We examined a seven-locus MLST (*aflG/aflL*, *aflV/aflW*, *aflW/aflX*, *mfs*, *trpC*, *MAT*, and *AF17*) for 64 *A. flavus* isolates that grouped into 47 VCGs. This included 59 isolates from a

Figure 2.6. Genetic connectivity between experimental F1 and natural *A. flavus* populations based on variation at seven MLST loci: *aflG/aflL*, *aflV/aflW*, *aflW/aflX*, *mfs*, *MAT*, *trpC* and AF17. The histogram bars indicate the number of isolates contained within a particular haplotype (see Table S2.8), and the numbers to the right of the blue bars in the natural population denote the vegetative compatibility groups (VCGs) represented within the haplotype. VCG testing was not done on the experimental population. Lines are used to connect haplotypes that are identical at the seven MLST loci between the experimental and natural populations. Asterisks indicate a natural haplotype that includes a parental isolate used in our mating studies; the parental haplotypes without connecting lines yielded only recombinant progeny. Seven F1 haplotypes are identical in sequence to one of their respective parental genotypes, and two F1 haplotypes match non-parental genotypes in nature (thickened lines). Natural haplotype H8 (IC244) matches F1 haplotype H59 that contains both a progeny (IC2202) and a non-progeny (IC1648) isolate. Natural haplotype H30 (IC294) is identical to F1 haplotype H46 that includes non-progeny isolates IC2167, IC2170 and IC2173.



single field population in Herod, Georgia, 3 isolates from peanut field soils of southern United States (IC310, IC311 and IC313), NRRL 21882 from Terrell County in Georgia and AF36 from Arizona. We found that the MLST uniquely fingerprinted 36 out of 44 VCGs from Georgia, 1 from Texas (VCG 76; Chang *et al.*, 2005) and another from Arizona (YV36; Ehrlich *et al.*, 2007) (Fig. 2.6; Table S8). We observed decoupling of VCG and MLST for VCG 24, which was split into H2 (NRRL 21882) and H32 (IC253), and for VCG 25 found in H9 (IC258) and H12 (IC259, IC260). For VCG 24, the decoupling was based on amplification and sequencing of *aflW/aflX*, *aflA*, *aflB*, *aflR* and *aflS* in IC253 which were not consistently amplified in NRRL 21882 as reported previously (Moore *et al.*, 2009); all other loci (*MAT*, *trpC*, *mfs* and AF17) were identical. For VCG 25, only variation in the AF17 microsatellite locus was responsible for separating vegetatively compatible strains into different MLSTs. The same seven-locus MLST inferred 59 unique progeny haplotypes from nine laboratory crosses, with 33 haplotypes represented by a single progeny, and 26 haplotypes containing two or more strains. The high degree of correspondence of this seven-locus MLST with VCGs in the natural population suggests that at least 86% of the progeny belong to new VCGs in the F1 experimental population. As expected, there was high haplotype diversity ($H_S = 0.983583$) in the experimental F1 and natural populations. Recombination rate estimates based on variation in *aflG/aflL*, *aflV/aflW*, *aflW/aflX*, and *mfs* regions were similar in experimental crosses ($\gamma = 0.002487$) and in the natural population ($\gamma = 0.002669$).

Progeny strains from a single cross often differed significantly from their parents in AF and CPA production (Table 2.2, see footnote 4). For example, progeny IC2205 from the

cross IC244 × IC277 showed evidence of crossing over on chromosome 3 (Fig. S2.1; Table 2.2) and exhibited significantly ($P \leq 0.05$) different total AF concentrations compared to those of either parent. This difference in AF production between progeny and parents was also observed when the cluster MLST was identical between a progeny strain and one of its parents; for example, total AF production in progeny IC2201 and the parent IC244. Furthermore, a significant difference in AF production was also observed when the genome MLST was shared between a progeny isolate and its corresponding parent; for example, total AF production in progeny IC2194 and the parent IC278.

There were no cases in which AF production in any of the progeny was found to be significantly higher than both parents; however, two progeny isolates (IC2193 and IC2204) were found to produce significantly higher amounts of CPA compared to both of their respective parents (Table 2.2). Progeny strains in cross IC311 × IC277 have significantly different CPA concentrations compared to either parent but were not well differentiated by either cluster or genome MLST. In the case of progeny strain IC1766, the hybridization signal intensity in the parentage plot for the subtelomeric segment of chromosome 3R (Fig. S2.1D) compared to the signal intensity elsewhere in the genome was very similar to the pattern observed in chromosome 3R for IC2209. This suggests that IC1766 may be heterokaryotic, which may explain the significant deviation of progeny CPA concentrations from either parent.

Discussion

We present in this study the first direct genetic evidence that sexual recombination is occurring in *A. flavus* through the meiotic processes of independent assortment and crossing over. We also have direct genetic evidence of crossovers influencing the toxin phenotype of *A. flavus* strains. For example, progeny strain IC2171 is the result of a crossover in IC301 (Table 2.2; Figs. 2.3, S2.1F) such that it gained *aflC* from AF36, making it AF- due to the nonsense mutation. In other cases crossovers do not result in a complete loss of toxicity but can significantly reduce AF production. For example, progeny strain IC1650 is the result of a crossover in IC278 such that it obtained the *aflL* to *aflP* region from AF36 (Fig. 2.3), which resulted in a significant reduction in toxicity (Table 2.2). We also have evidence of a progeny isolate gaining the AF+ phenotype. For example, progeny strain IC2205 has the genetic background of chromosome 3 from its AF- parent (IC277); however a crossover in the *aflK* region results in the gain of cluster regions between *aflF* to *aflQ* from its AF+ parent (IC244) (Figs. 2.3, S2.1G), resulting in progeny strain IC2205 being AF+ (Table 2.2); these data also indicate that mutations resulting in loss of AF production in IC277, which have yet to be determined, reside between *aflF* and *aflQ*. Although we did not observe a progeny isolate that had the genetic background of one parent and chromosome 3 of the other parent via independent assortment, we would expect to detect this with a larger sampling of offspring.

Our experimental results are consistent with indirect inferences of recombination from genealogical studies of natural populations of AF+ fungi (Carbone *et al.*, 2007; Geiser *et al.*, 1998; Moore *et al.*, 2009; Peterson *et al.*, 2001). Furthermore, crossover breakpoints

within the AF cluster of recombinant progeny corroborate with breakpoints deduced from the genetic analysis of natural populations (Moore *et al.*, 2009), which suggests that certain regions in the cluster are more prone to recombination (Fig. 2.3). The recovery of conserved breakpoints and shared MLSTs (Fig. 2.6) in experimental progeny strongly suggests that recombination in nature is occurring between different VCGs and on a contemporary time scale. This process should not be confused with balancing selection acting on the AF-phenotype in *A. flavus* (Moore *et al.*, 2009), which results in maintenance of ancestral polymorphisms in cluster genes; in the presence of recombination this may be confounded with cryptic speciation (Geiser *et al.*, 1998) and incomplete lineage sorting (Grubisha, Cotty, 2009a). Overall, the results of this study show that a single round of sexual reproduction in *A. flavus* can generate contemporary patterns of recombination and account for VCG and mycotoxin diversity.

The results of experimental matings indicate that the genetic and functional diversity observed in *A. flavus* progeny is the result of independent assortment of chromosomes in parents coupled with intra- and interlocus crossover events, all of which are hallmarks of meiosis. The high MLST diversity among progeny in *A. flavus* is consistent with our observations in nature that most of the individuals are vegetatively incompatible and have different MLSTs (see Fig. 2.6). Several shared MLSTs exist between our experimental F1 and natural populations. While the majority of these shared genotypes are between a progeny isolate and its respective parent, there are also matching MLSTs between F1 progeny and non-parental natural isolates (denoted as bold lines in Fig. 2.6). The recovery of shared MLSTs suggests that processes of independent assortment and crossing over detected

in experimental progeny are also occurring in nature. The accurate reconstruction of recombinant progeny and their respective parents in the ARG (Fig. 2.5) further reinforces the long history of recombination inferred in natural populations (see Fig. 2.2 in Moore *et al.*, 2009). The ARG also shows that AF36 in haplotype H2 has undergone both mutation and recombination in its recent past (Fig. 2.5).

Because AF biosynthesis is polygenic, the interaction of genes from other chromosomes may influence AF production beyond mutation and recombination events in the AF cluster *per se*. Estimates of heritability show that both AF and CPA are highly heritable (Fig. 2.1). This indicates that the majority of the genetic variation in mycotoxin production arises from mutations in AF cluster genes such that highly toxigenic parents produce progeny that are also highly toxigenic. For example, both the parental and F1 progeny strains in cross IC308 × IC307 had the highest midpoint and mean AF and CPA concentrations among the crosses (Fig. 2.1). A more extensive sample of progeny in this cross might detect causal mutations in the AF cluster that result in increased toxicities. At the other extreme, cross IC310 × NRRL 21882 was AF- for both parents and offspring (Fig. 2.1). In previous work, our examination of molecular sequence variation across 21 intergenic regions in the AF gene cluster of *A. flavus* (Moore *et al.*, 2009) indicates that balancing selection is acting on AF- phenotypes with partial or completely missing AF cluster genes; this selection presumably also maintains the heritability observed in laboratory crosses (Barton, 1990; Barton, Keightley, 2002). The implication is that in the absence of sex, selection will maintain non-aflatoxicity in *A. flavus* populations, whereas in the presence of sex, high AF heritability will shift populations in favor of increasing aflatoxicity.

The genetic continuity observed between the F1 experimental and natural populations suggests that sexual reproduction accounts for a significant proportion of the genetic and VCG diversity in field populations, as well as quantitative variation in mycotoxin production. The near 1:1 distribution ratio of *MATI-1*:*MATI-2* isolates in the experimental (Table 2.2) and the corresponding natural population (Moore *et al.*, 2009; Ramirez-Prado *et al.*, 2008) is consistent with sexual reproduction; asexual populations generally show strong deviation from this ratio (Paoletti *et al.*, 2005). Preliminary evidence from sampling geographically isolated *A. flavus* populations indicates that when the frequencies of *MATI-1* and *MATI-2* are not significantly different, the mean toxin concentrations are significantly higher than the levels in populations with skewed mating type ratios (Moore, 2010).

The vertical transmission of cryptic alleles suggests that while *A. flavus* is predominantly homokaryotic, the species may harbor common or rare alleles at a low copy number. A specific case in point is the DNA sequence of the *aflG/aflL* locus in IC1716 that matches the consensus DNA sequence of 63 isolates (59 from Georgia, 1 from NC [IC310], 1 from Texas [IC313], 1 from Alabama [IC311] and 1 from Arizona [AF36]) of *A. flavus* sampled from natural populations (Tables S2.2 and S2.3). Because the consensus sequence is based on the most frequent segregating base at each polymorphic site, it represents the putative ancestral lineage in the sample. This effectively rules out contamination at the DNA sequence level as a possible explanation for the inheritance of parental alleles from other crosses, and suggests that *A. flavus* strains retain a genetic cache of cluster genes, which is possibly of adaptive significance. Cryptic alleles were only detectable in AF cluster genes between parents with partial or completely missing AF gene clusters (Table 2.2). For

example, the AF cluster regions that were amplified and sequenced in IC2209 matched the parent strain IC277 that is a CPA producer even though IC2209 is non-toxicogenic and is missing the entire subtelomeric region of chromosome 3R according to the aCGH (Fig. 2.2). It is possible that cryptic alleles are found elsewhere in the genome but without additional cloning, low copy number alleles would be masked by the dominant copies. A similar phenomenon of inheritance of non-parental ancestral alleles has been reported in *Saccharomyces cerevisiae* (Nevzglyadova *et al.*, 2001), *Arabidopsis* (Lolle *et al.*, 2005) and most recently in *Phytophthora ramorum* (Vercauteren *et al.*, 2011).

We hypothesize that some *A. flavus* strains are heterokaryotic for the presence and absence of the AF gene cluster. A specific case in point is offspring strain IC1766, which appears to be heterokaryotic in chromosome 3R (Fig. 2.3). In this example, heterokaryosis may be responsible for the decoupling of CPA production and the AF cluster haplotype in the cross IC311 × IC277. Heterokaryosis is also supported by aCGH data and amplification/sequencing of cluster genes in IC2209 and partial cluster parents (Table 2.2; Moore *et al.*, 2009). The exact mechanism underlying AF cluster gene deletions is unknown; however, sharp transitions in G + C content and high recombination activity may be accelerating gene loss in *A. flavus* (Moore *et al.*, 2009). Partial and entire deletions of AF cluster genes have been reported in *A. flavus* (Chang *et al.*, 2005; Jiang *et al.*, 2009; Moore *et al.*, 2009) and *A. oryzae* (Kusumoto *et al.*, 2000; Lee *et al.*, 2006). Comparative synteny analysis between *A. flavus* NRRL 3357 (GenBank accession scaffold EQ963481.1) and *A. oryzae* NRRL 5590 (GenBank accession scaffolds AP007169 and AP007170) genome strains has revealed an approximate 1-Mb subtelomeric translocation from chromosome 2 to 6 in *A.*

oryzae (R. A. Olarte, unpublished data). Deletions may be associated with simultaneous duplications or translocations of other genomic regions (Smith *et al.*, 2007) but the precise chromosomal location of translocated regions cannot be ascertained using aCGH alone. For example, a heat map comparing IC2209 and the NRRL 3357 *A. flavus* reference strain on the array revealed an approximately 1.1-Mb subtelomeric duplication starting with predicted gene 2504.m00371 (tropomyosin TPM1) on chromosome 1 (J. T. Monacell, unpublished data); whether this duplication has been translocated to chromosome 3R is unknown. All of the *A. flavus* deletion strains examined using aCGH in this study confirm deletion patterns reported previously, and also indicate that deleted cluster genes have not been translocated to other chromosomes. Because *A. flavus* is multinucleate, we would expect aCGH to detect inter-nuclear translocation events, suggesting that cryptic alleles are from nuclei that are at a low copy number. This would give rise to genetically unbalanced nuclei where the majority of nuclei have full AF gene clusters and a small minority have partial or full deletions of the cluster, or vice-versa. This would explain why cryptic cluster alleles, present in less than single copy amounts in partial cluster parents and progeny in this study (Table 2.2), are only detectable via PCR amplification and sequencing. Whether these low frequency cluster alleles play a role in regulation of AF production is unknown. Future research will focus on elucidating the mechanism and origins of these cryptic alleles.

Silencing of AF cluster genes in *A. flavus* may occur through transvection or trans-sensing mechanisms (Smith *et al.*, 2007; Woloshuk *et al.*, 1995). These terms have been used to describe the phenomenon whereby wild-type genes are inactivated due to unpaired alleles as a result of rearrangements, deletions or translocations at a locus. In *A. flavus*, the

repression of expression is thought to occur during synapsis in meiosis or possibly mitotic pairing associated with the parasexual cycle. For example, a diploid formed between a wild-type *A. flavus* strain and mutant strain 649 (317-kb deletion of chromosome 3R and replaced with a 939-kb duplication of the end of chromosome 2R) is non-toxigenic (Smith *et al.*, 2007); adding an ectopic copy of *aflR* restores toxicity, which suggests that a single copy of *aflR* can activate silenced genes (Smith *et al.*, 2007). Since all partial and full cluster deletions are missing *aflR*, the insertion of a single ectopic copy of *aflR* in these strains may influence the overall toxin phenotype by relieving silencing. For example, this may restore AF toxicity in IC2209, which is missing 374 kb from the end of chromosome 3R but harbors the parental (IC277) CPA and AF cluster genes at low copy number; this is currently under investigation.

Biological control is an effective means of reducing AF contamination in crops; however, the long-term effect of AF- biocontrol strains on native populations needs to be reassessed with the recent discovery of the sexual cycle in aflatoxigenic fungi (Horn *et al.*, 2009a; Horn *et al.*, 2011; Horn *et al.*, 2009b; Horn *et al.*, 2009c). The biocontrol strains AF36 and NRRL 21882 were isolated from nature and are similar to many other AF- strains in competitiveness (Atehnkeng *et al.*, 2008; Horn, Dorner, 2011), with the main factor in their competitiveness being their high population numbers relative to those of native AF+ strains. Application of a single biocontrol strain to fields greatly increases the population density of a single genotype that consists of one mating type, which in the case of AF36 and NRRL 21882, is *MATI-2* (Table 2.2). The high population density of the biocontrol strain would likely increase the frequency of sexual reproduction, which would largely comprise

matings between the biocontrol strain and native *MAT1-1* strains. Given the high heritability of AF and CPA and the relative ease of gaining or losing toxicity via crossovers and independent assortment in a single generation of sex, sexual reproduction could play a role in the persistence of mycotoxins in fields in which these biocontrol strains are applied (Cotty, 1994; Dorner *et al.*, 1999). Application of AF36 or NRRL 21882 to fields for AF control, especially when applied repeatedly over many years, could result in a shift in the genetic composition of native strains due to the transfer of genes from the biocontrol strain. The capacity of these new genotypes to produce mycotoxins and their competitiveness in invading crops merit close evaluation.

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Data Accessibility:

-DNA sequences: Genbank accessions JF418181 – JF418920

-aCGH data: Gene Expression Omnibus (GEO) accessions GSM679190 – GSM679207

Rodrigo Olarte is a PhD student interested in understanding the ramifications of sexual recombination in experimental and natural populations of *A. flavus*. Bruce Horn is a Research Microbiologist interested in the ecology and population biology of aflatoxigenic fungi. Joe Dorner is a Research Microbiologist (retired) who developed the Afla-Guard[®] biocontrol formulation. James “Trent” Monacell is a PhD student interested in the genomics of filamentous fungi. Rakhi Singh is interested in the molecular biology of fungi. Eric Stone is interested in developing statistical methods for analyzing genomic data. Ignazio Carbone’s laboratory focuses on the population genomics of agriculturally important *Aspergillus* species.

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APPENDICES

Appendix A

Table S2.1. Predicted genes on the right arm of chromosome 3 of *Aspergillus flavus*

Protein ID	Genbank Accession	TIGR gene prediction
2911.m00826	EED51201.1	Nonribosomal peptide synthase, putative
2911.m00825	EED51200.1	Hypothetical protein
2911.m00824	EED51199.1	Hypothetical protein
2911.m00823	EED51198.1	Hypothetical protein
2911.m00822	EED51197.1	ABC multidrug transporter, putative
2911.m00821	EED51196.1	Amino acid permease family protein
2911.m00820	EED51195.1	Conserved hypothetical protein
2911.m00819	EED51194.1	Hypothetical protein
2911.m00818	EED51193.1	Hypothetical protein
2911.m00817	EED51192.1	Gluconolactone oxidase, putative
2911.m00816	EED51191.1	Hypothetical protein
2911.m00815	EED51190.1	Fungal specific transcription factor domain containing protein
2911.m00814	EED51189.1	Lignostilbene dioxygenase family protein
2911.m00813	EED51188.1	Hypothetical protein
2911.m00812	EED51187.1	Amidohydrolase family protein
2911.m00811	EED51186.1	Serine protease prots-related
2911.m00810	EED51185.1	Hypothetical protein
2911.m00809	EED51184.1	Fungal specific transcription factor domain containing protein
2911.m00808	EED51183.1	NAD dependent epimerase/dehydratase family protein (<i>pks/nrps</i>)
2911.m00807	EED51182.1	Dimethylallyl tryptophan synthase, putative (<i>dmaT</i>)
2911.m00806	EED51181.1	Hypothetical protein (<i>maoA</i>)
2911.m00805	EED51180.1	Major facilitator superfamily protein (<i>mfs1</i>)
2911.m00804	EED51178.1	<i>aflF</i> / <i>norB</i> / dehydrogenase aflatoxin
2911.m00803 ¹	EED51177.1 ¹	<i>aflU/cypA</i> /P450 monooxygenase (43-340) ¹
2911.m00802 ¹	EED51177.1 ¹	<i>aflU/cypA</i> /P450 monooxygenase (341-603, 697-1038) ¹
2911.m00801	EED51176.1	<i>aflT</i> / <i>aflT</i> / transmembrane protein aflatoxin
2911.m00800	EED51175.1	<i>aflC</i> / <i>pksA</i> / <i>pksL1</i> / polyketide synthase aflatoxin
2911.m00799	NA ^{2,3}	Hypothetical protein
2911.m00798	EED51174.1	<i>aflCa</i> / <i>hypC</i> / hypothetical protein aflatoxin
2911.m00797	EED51173.1	<i>aflD</i> / <i>nor-1</i> / reductase aflatoxin
2911.m00796	EED51172.1	<i>aflA</i> / <i>fas-2</i> / <i>hexA</i> / fatty acid synthase alpha subunit aflatoxin
2911.m00795	EED51171.1	<i>aflB</i> / <i>fas-1</i> / fatty acid synthase beta subunit aflatoxin

Table S2.1. Continued

2911.m00794	EED51170.1	<i>aflR</i> / <i>apa-2</i> / <i>afl-2</i> / transcription activator aflatoxin
2911.m00793	EED51169.1	<i>aflS</i> / pathway regulator aflatoxin
2911.m00792	EED51168.1	Aflatoxin biosyntheses short-chain alcohol dehydrogenases AdhA
2911.m00791	EED51167.1	<i>aflJ</i> / <i>estA</i> / esterase aflatoxin
2911.m00790	EED51166.1	<i>aflE</i> / <i>norA</i> / <i>aad</i> / <i>adh-2</i> / NOR reductase/ dehydrogenase aflatoxin
2911.m00789	EED51165.1	<i>aflM</i> / <i>ver-1</i> / dehydrogenase/ ketoreductase aflatoxin
2911.m00788	EED51163.1	<i>aflN</i> / <i>verA</i> / monooxygenase aflatoxin
2911.m00787	EED51162.1	<i>aflNa</i> / <i>hypD</i> / hypothetical protein aflatoxin
2911.m00786	EED51161.1	<i>aflG</i> / <i>avnA</i> / <i>ord-1</i> / cytochrome P450 monooxygenase aflatoxin
2911.m00785	EED51160.1	<i>aflL</i> / <i>verB</i> / desaturase/ P450 monooxygenase aflatoxin
2911.m00784	EED51159.1	<i>aflLa</i> / <i>hypB</i> / hypothetical protein aflatoxin
2911.m00783	EED51158.1	Aflatoxin biosynthesis averufin dehydrogenase <i>AyfA</i>
2911.m00782	EED51157.1	<i>aflO</i> / <i>omtB</i> / <i>dmtA</i> / O-methyltransferase B aflatoxin
2911.m00781	EED51156.1	<i>aflP</i> / <i>omtA</i> / <i>omt-1</i> / O-methyltransferase A aflatoxin
2911.m00780	EED51155.1	<i>aflQ</i> / <i>ordA</i> / <i>ord-1</i> / oxidoreductase/ cytochrome P450 monooxygenase aflatoxin
2911.m00779	EED51154.1	<i>aflK</i> / <i>vbs</i> / VERB synthase aflatoxin
2911.m00778	EED51153.1	<i>aflV</i> / <i>cypX</i> / cytochrome P450 monooxygenase aflatoxin
2911.m00777	EED51152.1	<i>aflW</i> / <i>moxY</i> / monooxygenase aflatoxin
2911.m00776	EED51151.1	<i>aflX</i> / <i>ordB</i> / monooxygenase/ oxidase aflatoxin
2911.m00775	EED51150.1	<i>aflY</i> / <i>hypA</i> / <i>hypP</i> / hypothetical protein aflatoxin
2911.m00774	EED51149.1	<i>aflYa</i> / <i>nadA</i> / NADH oxidase aflatoxin
2911.m00773	NA ^{3,4}	NADH oxidase, putative (<i>hxtA</i>)
2911.m00772	EED51148.1	Hexose transporter, putative (<i>glcA</i>)
2911.m00771	EED51147.1	Alpha-glucosidase/alpha-amylase, putative (<i>sugR</i>)
2911.m00770	EED51146.1	Transcriptional regulator, putative
2911.m00769	EED51145.1	Ser/Thr protein phosphatase family protein
2911.m00768	EED51144.1	Major facilitator superfamily protein
2911.m00767	EED51143.1	ATPase, AAA family protein
2911.m00766	EED51142.1	Hypothetical protein
2911.m00765	EED51141.1	ATPase, AAA family protein
2911.m00764	EED51140.1	Hypothetical protein
2911.m00763	EED51139.1	Hypothetical protein
2911.m00762	EED51138.1	Major facilitator superfamily protein
2911.m00761	EED51137.1	Hypothetical protein

Table S2.1. Continued

2911.m00760	EED51136.1	Sterol 4-a-methyl-oxidase-related
2911.m00759	EED51135.1	Hypothetical protein
2911.m00758	EED51134.1	Hypothetical protein
2911.m00757	EED51133.1	Oxidoreductase, FAD/FMN-binding family protein
2911.m00756	EED51132.1	Hypothetical protein
2911.m00755	EED51131.1	Oxidoreductase, short chain dehydrogenase/reductase family protein
2911.m00754	EED51130.1	Oxidoreductase, zinc-binding dehydrogenase family protein
2911.m00753	EED51129.1	Flavin-binding monooxygenase-like family protein
2911.m00752	EED51128.1	Fungal-specific transcription factor domain containing protein
2911.m00751	EED51127.1	Flavin-binding monooxygenase-like family protein
2911.m00750	EED51126.1	Metallo-beta-lactamase superfamily protein
2911.m00749	EED51125.1	Major facilitator superfamily protein
2911.m00748	EED51124.1	Hypothetical protein
2911.m00747	EED51123.1	Protein kinase domain containing protein
2911.m00746	EED51122.1	Conserved hypothetical protein
2911.m00745	EED51121.1	Glycosyl hydrolases family 16 protein

¹The gene models for 2911.m00802 and 2911.m00803 were merged together under the same EED51177.1 Genbank accession. The corresponding spans within the TIGR gene prediction (*aflU/cypA/P450* monooxygenase) are indicated within parentheses.

²The gene model for 2911.m00779 was deleted because it was merged with the neighboring 2911.m00800 (*aflC / pksA / pksL1 / polyketide synthase aflatoxin*).

³NA = not available.

⁴The gene model for 2911.m00773 was deleted because it was merged with the neighboring 2911.m00774 (*aflYa/ nadA/ NADH oxidase aflatoxin*).

Appendix B

Table S2.2. Distribution of haplotypes and base substitutions in the *aflG/aflL* intergenic region of 63 natural *Aspergillus flavus* isolates, including AF36, plus progeny isolate IC1716

Locus	aflG/aflL
Position in combined consensus	11222222222222333333333333334444 23566701122355566901112334567990345 58180874859427838221287340292069101
Site Number	11111111112222222222333333 12345678901234567890123456789012345
Site Type	tvtvttttvtvtvtvtvtvtvtvtvtvtvtvtvtvtvt
Character Type	-----i-i--i-iiii-i--i--i-iii-ii-i
Consensus	GGGTCCGCAGTGGTGTCAACGGTACTCTGGCCATC
Haplotype (Frequency)	
H1 (2)C.....G.....
H2 (1)	A.....G..A.....CA.....
H3 (1)	.C.G...T...C.ACT..T..CC.A.C.A.TT.T
H4 (1)	..A.T.A.....C.C.....G.GC.....
H5 (1)T.....C.
H6 (34)T...C.ACT..T..C..A.C.A.TT.T
H7 (5)G..A.....
H8 (6)C.....G....
H9 (9)C.....
H10 ¹ (1)
H11 (2)G.....CA.....
H12 (1)A.....

t, transitions; v, transversions; i, phylogenetically informative sites; -, uninformative sites

¹Haplotype H10 contains progeny isolate IC1716.

Appendix C

Table S2.3. Haplotypes and strain designations for the *aflG/aflL* intergenic region haplotype distribution map shown in Table S2.2

Haplotype	Strain
H1	AF36, IC289
H2	IC310
H3	IC303
H4	IC313
H5	IC311
H6	IC234, IC235, IC236, IC244, IC258, IC259, IC260, IC267, IC268, IC269, IC270, IC271, IC272, IC273, IC274, IC275, IC278, IC280, IC281, IC282, IC284, IC287, IC290, IC292, IC293, IC294, IC295, IC297, IC298, IC299, IC300, IC301, IC302, IC304
H7	IC245, IC246, IC247, IC253, IC307
H8	IC229, IC230, IC231, IC279, IC286, IC306
H9	IC263, IC264, IC265, IC276, IC283, IC285, IC288, IC291, IC305
H10	IC1716
H11	IC277, IC308
H12	IC296

Table S2.4. Continued

H15 (1)T.G.....A....	C.T...G.....	TC..G..T.....C.1
H16 (1)T.G.....A....	C.T...G.....	TC..G..T.....G1...	.C.1
H17 (1)T.G.....A....	C.T...G.....	TC..G..T.....	1 G1...	.C.1
H18 (1)	..A..C...G.....A....C.....	1 G1...	...1
H19 (6)	..A..C...G.....A....	.T...G.....	TC.G.AA.CCGAC.GTCGT.AAAGATAGTCCTGCACGATGCCCGC.TAA.CGTTC	...2A.	1	T.C.
H20 (4)	..A..C...G.....A....	.T...G.....	TC.G.AA.CCGAC.GTCGT.AAAGATAGTCCTGCACGATGCCCGC.TAA.CGTTC	...2A.	1 G1...	.C..
H21 (1)	..A..C...G.....A....	.T...G.....	TC.G.AA.CCGAC.GTCGT.AAAGATAGTCCTGCACGATGCCCGC.TAA.CGTTC	...2A.	1 G1...	...1
H22 (1)	..A..C...G.....A....	.T...G.....	TC.G.AA.CCGAC.GTCGT.AAAGATAGTCCTGCACGATGCCCGC.TAA.CGTTC	...2A.	11
H23 (1)	..A..C...G.....A....	.T...G.....	TC.G.AA.CCGAC.GTCGT.AAAGATAGTCCTGCACGATGCCCGC.TAA.CGTTC	...2A.	. G1...	.C..
H24 (1)	..A..C...G.....A....	.T...G.....	TC.G.AA.CCGAC.GTCGT.AAAGATAGTCCTGCACGATGCCCGC.TAA.CGTTC	...2A.	. G1...	...1
H25 (1)	..A..C...G.....A....	.T...G.....	TC.G.AA.CCGAC.GTCGT.AAAGATAGTCCTGCACGATGCCCGC.TAA.CGTTC	...2A.1
H26 (1)	..C.A.....	..CG..AT.....T.....A.....	A2....	. G1...	.C.1
H27 (1)	..C.A.....	..CG..AT.....T.....	C.T...G.....	TC..G..T..... G1...	.C.1
H28 (2)	..C.A.....	..CG..AT.....T.....A.....	A2....	1 G1...	.C.1
H29 (1)	..C.A.....	..CG..AT.....T.....A.....	A2....	1 G1...	...1
H30 (2)	..C.A.....	..CG..AT.....T.....A.....	A2....1
H31 (1)	..C.A.....	.T.....CACT..T.CA..ATTT	.T...G.....	TC.G.AA.CCGAC.GTCGT.AAAGATAGTCCTGCACGATGCCCGC.TAA.CGTTC	...2A.	. G1...	T.C.
H32 (1)	..C.A.....	.T.....CACT..T.CA..ATTTT.	..C..... G1...	.C..
H33 (1)	..C.A.....	.T.....CACT..T.CA..ATTTT.....T	1 ..T.G	.C..
H34 (3)	..C.A.....	.T.....CACT..T.CA..ATTTT.....T	. ..T.G	.C..
H35 (1)	..C.A.....	.T.....CACT..T.CA..ATTTT.....TC..
H36 (2)	..C.A.....	.T.....CACT..T.CA..ATTTT.....T	1C..
H37 (3)	..C.A.....	.T.....CACT..T.CA..ATTTC..... G1...	.C..
H38 (2)	..C.A.....	.T.....CACT..T.CA..ATTTC.....C..
H39 (2)	..C.A.....	.T.....CACT..T.CA..ATTTC.....G.	...1
H40 (2)	..C.A.....	.T.....CACT..T.CA..ATTTC.....G.	.C..
H41 (3)	..C.A.....	.T.....CACT..T.CA..ATTTC.....1
H42 (3)	..C.A.....	.T.....CACT..T.CA..ATTTC..... G1...	...1
H43 (1)	..C.A.....	.T.....CACT..T.CA..ATTTC.....	1 ...G.	...1
H44 (1)	..C.A.....	.T.....CACT..T.CA..ATTTC.....	11
H45 (2)	..C.A.....	.T.....CACT..T.CA..ATTTC.....	1	T.C.
H46 (1)	..C.A.....	.T.....CACT..T.CA..ATTTC.....	1C..
H47 (1)	..C.A.....	.T.....CACT..T.CA..ATTTC.....	1 G1...	T.C.
H48 (4)	..C.A.....	.T.....CACT..T.CA..ATTTC.....	1 G1...	.C..

t, transitions; v, transversions; i, phylogenetically informative sites; -, uninformative sites

Appendix E

Table S2.5. Haplotypes and strain designations in Table S2.4

Haplotype	Strain
H1	IC244, IC1648, IC2202
H2	AF36, IC2165, IC2168
H3	IC2171
H4	IC1646
H5	IC1652
H6	IC1647, IC1651, IC1654
H7	IC2172
H8	IC2174
H9	IC310
H10	IC1720
H11	IC1719
H12	IC277, IC2190, IC2208, IC2209
H13	IC308
H14	IC1740, IC1741, IC1747
H15	IC1750
H16	IC1745
H17	IC1749
H18	IC2207
H19	IC1765, IC1768, IC1772, IC1773, IC1774, IC2200
H20	IC2189, IC2192, IC2193, IC2197
H21	IC2204
H22	IC2212
H23	IC2196
H24	IC2211
H25	IC2210
H26	IC307
H27	IC1751
H28	IC1743, IC1748
H29	IC1744
H30	IC1742, IC1746
H31	IC2205

Table S2.5. Continued

H32	IC1650
H33	IC301
H34	IC2167, IC2170, IC2173
H35	IC2169
H36	IC2166, IC2176
H37	IC1645, IC1655, IC1671
H38	IC1649, IC1653
H39	IC1669, IC1672
H40	IC1670, IC1673
H41	IC1700, IC2201, IC2206
H42	IC1679, IC1692, IC1694
H43	IC1677
H44	IC1696
H45	IC2191, IC2203
H46	IC2195
H47	IC2198
H48	IC278, IC1644, IC2194, IC2199

Appendix G

Table S2.7. Haplotypes and strain designations for the *A. flavus* ARG in Figure 2.5

Haplotype	Strain
H1	IC277, IC1765, IC1768, IC1772, IC1773, IC1774, IC2189, IC2190, IC2192, IC2193, IC2196, IC2197, IC2200, IC2204, IC2208, IC2209, IC2210, IC2211, IC2212
H2	AF36, IC1646, IC1647, IC1651, IC1652, IC1654, IC2165, IC2168, IC2172, IC2174
H3	IC2171
H4	IC310, IC1719, IC1720, IC1722
H5	IC244, IC278, IC1644, IC1645, IC1648, IC1649, IC1653, IC1655, IC1669, IC1670, IC1671, IC1672, IC1673, IC1677, IC1679, IC1692, IC1694, IC1696, IC1700, IC2191, IC2194, IC2195, IC2198, IC2199, IC2201, IC2202, IC2203, IC2206
H6	IC307, IC1742, IC1743, IC1744, IC1746, IC1748
H7	IC1751
H8	IC2205
H9	IC1650
H10	IC301, IC2166, IC2167, IC2169, IC2170, IC2173, IC2176
H11	IC308, IC1740, IC1741, IC1745, IC1747, IC1749, IC1750
H12	IC2207

Appendix H

Table S2.8. Haplotypes and strain designations in Figure 2.6

F1 population	
Haplotype	Strain
H1	IC1644, IC2194, IC2199
H2	IC1693, IC1703
H3	IC2209
H4	IC1695, IC1697, IC1698, IC1701
H5	IC1717, IC1723
H6	IC1725, IC1726, IC1727
H7	IC1699
H8	IC1716, IC1718, IC1721
H9	IC1724
H10	IC1719
H11	IC1720
H12	IC1722
H13	IC1668
H14	IC1676
H15	IC1678
H16	IC1675
H17	IC1674
H18	IC1764, IC1766, IC1767, IC1769, IC1770, IC1775
H19	IC1771
H20	IC1646
H21	IC1742, IC1746
H22	IC1751
H23	IC1743, IC1748
H24	IC1744
H25	IC1740, IC1741, IC1747
H26	IC1765, IC1768, IC1772, IC1773, IC1774, IC2200
H27	IC2207
H28	IC2190, IC2208
H29	IC2196

Table S2.8. Continued

H30	IC2211
H31	IC2210
H32	IC2189, IC2192, IC2193, IC2197
H33	IC2204
H34	IC2212
H35	IC1749
H36	IC1750
H37	IC1745
H38	IC2165, IC2168
H39	IC2172
H40	IC2174
H41	IC1652
H42	IC1647, IC1651, IC1654
H43	IC2205
H44	IC1650
H45	IC2166, IC2176
H46	IC2167, IC2170, IC2173
H47	IC2169, IC2171
H48	IC1645, IC1655, IC1671
H49	IC1649, IC1653
H50	IC1669, IC1672
H51	IC1670, IC1673
H52	IC1700, IC2201, IC2206
H53	IC1679, IC1692, IC1694
H54	IC1677
H55	IC1696
H56	IC2191, IC2203
H57	IC2195
H58	IC2198
H59	IC1648, IC2202

Natural population

Haplotype	Strain
H1	IC229, IC230, IC231

Table S2.8. Continued

H2	NRRL 21882
H3	IC310
H4	IC303
H5	IC313
H6	IC311
H7	IC234, IC235, IC236
H8	IC244
H9	IC258
H10	IC292, IC304
H11	IC287
H12	IC259, IC260
H13	IC278
H14	IC274, IC275
H15	IC282
H16	IC295
H17	IC270, IC271, IC302
H18	IC293
H19	IC297
H20	IC298
H21	IC300
H22	IC301
H23	IC280
H24	IC281
H25	IC272, IC273
H26	IC284
H27	IC299
H28	IC290
H29	IC267, IC268, IC269
H30	IC294
H31	IC245, IC246, IC247, IC307
H32	IC253
H33	IC289
H34	AF36

Table S2.8. Continued

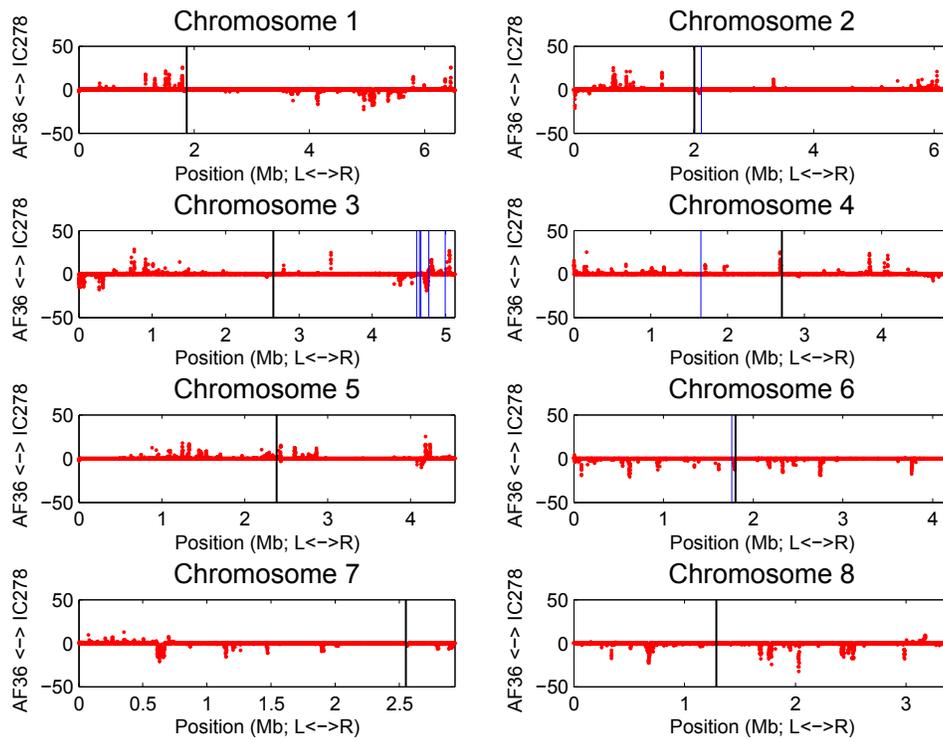
H35	IC277
H36	IC296
H37	IC308
H38	IC263, IC264, IC265
H39	IC288
H40	IC285
H41	IC291
H42	IC305
H43	IC283
H44	IC276
H45	IC306
H46	IC286
H47	IC279

Appendix I

Figure S2.1. Genome-wide parentage plots for the parent-offspring trio heat maps shown in Figures 2 and 3. Each of the plots labeled from A-I corresponds to one offspring. In the plots, a black vertical line indicates the centromere, and blue vertical lines indicate the locations of the loci used in our multilocus sequence typing (MLST). Microsatellite locus AF17 is represented on chromosome 2; AF cluster loci *aflC*, *aflG/aflL*, *aflV/aflW*, *aflW/aflX* and linked locus *mfs* are on chromosome 3; the *trpC* locus is on chromosome 4; and the mating-type locus (*MAT*) is on chromosome 6. Each red dot in the chromosome plot identifies one probe. On the x-axis is the approximate position of the probe on the chromosome in Mb; on the y-axis is the degree of similarity of the offspring to each parent, which is a score that predicts parental origin. Chromosomes showing different parental origins along their entire length in the progeny would be indicative of independent assortment. For example, in Figure S2.1A, a negative difference for most of the probes on chromosome 8 suggests that progeny strain IC1650 is more similar to AF36, which is the bottom parent in the plot, whereas a positive difference for the probes in chromosome 5 translates to IC1650 showing more similarity to IC278, the top parent in the plot. Alternating probe patterns indicate that a crossover has occurred. For example, at least one crossover can be discerned in the rightmost portion of chromosome 3R, which is corroborated with our genetic markers in the AF cluster.

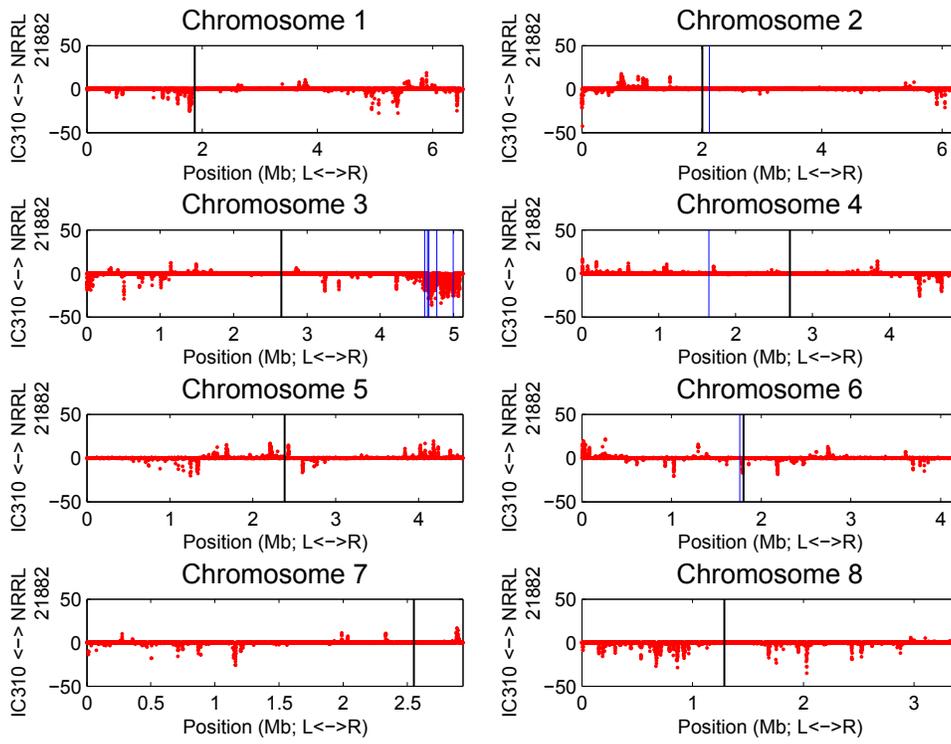
A

IC1650



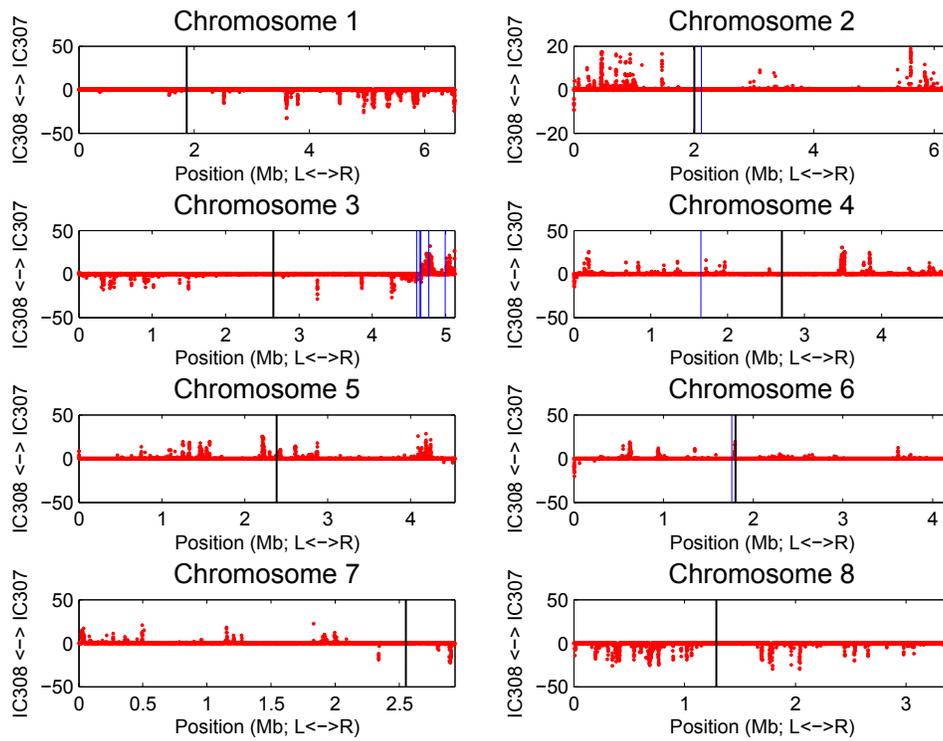
B

IC1719



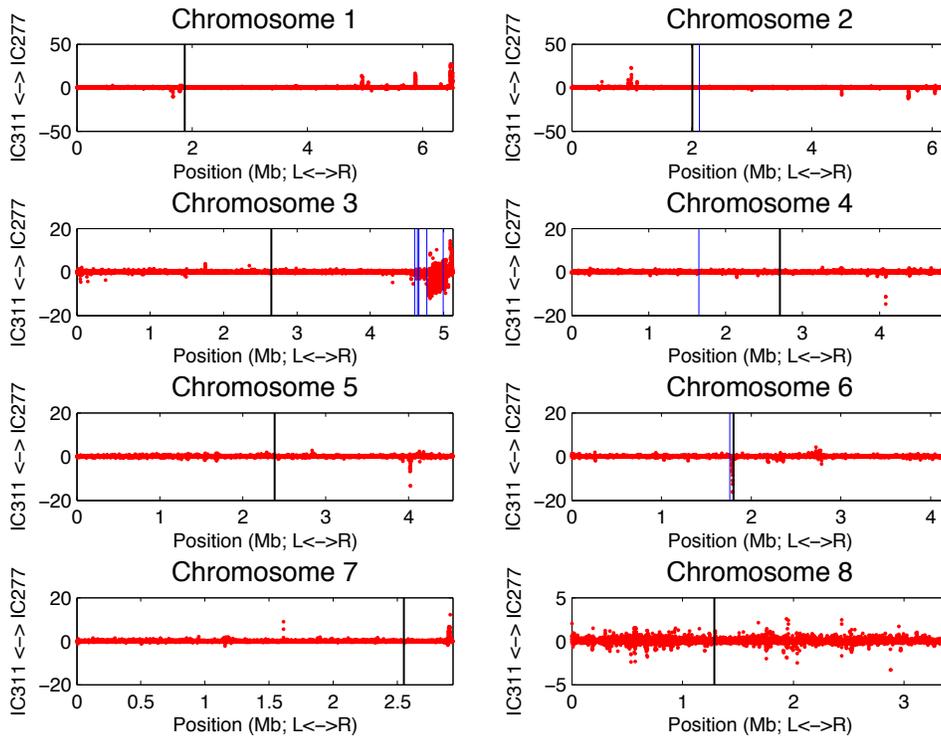
C

IC1751



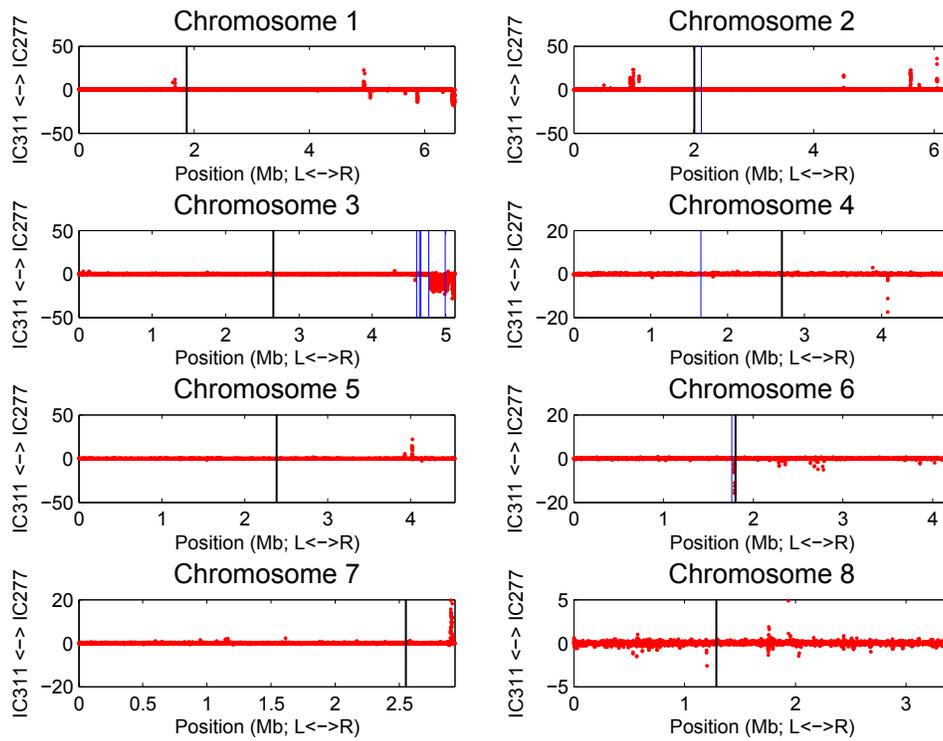
D

IC1766



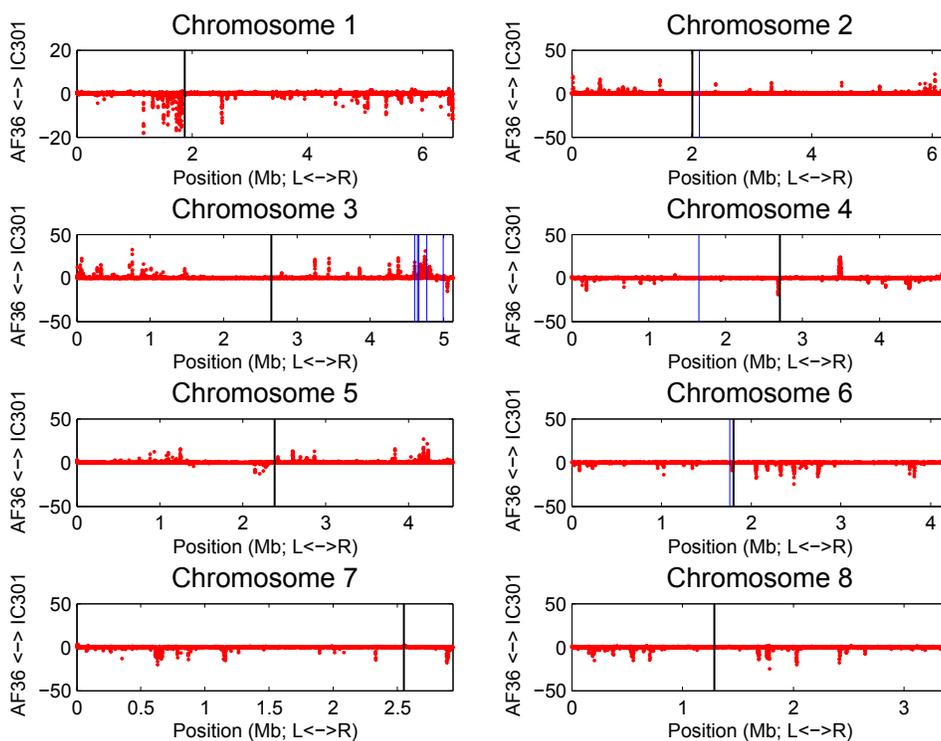
E

IC1775



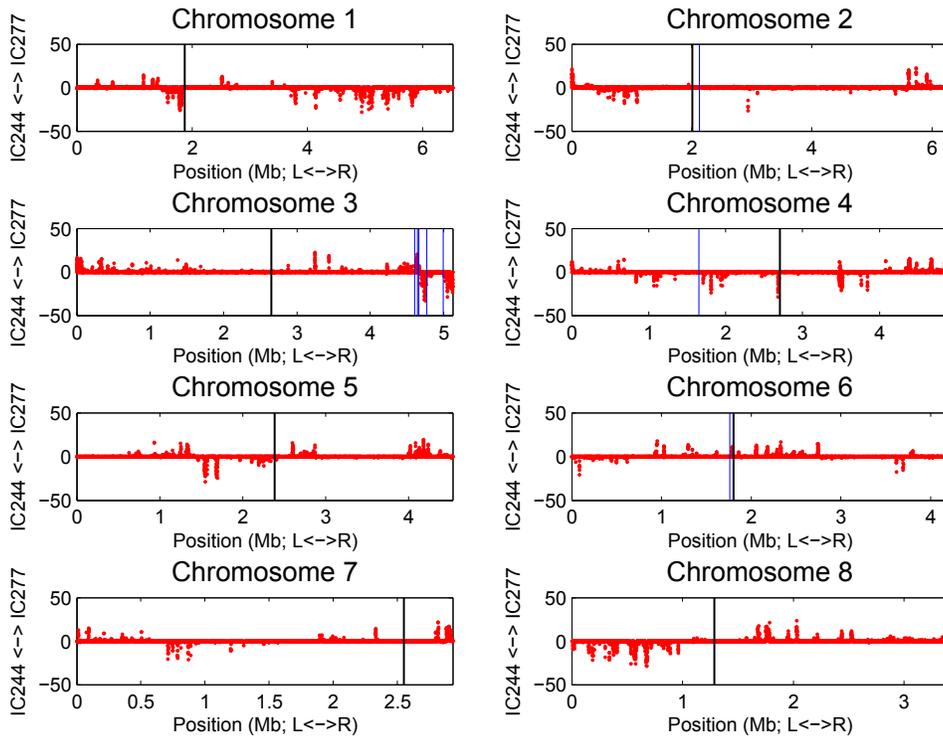
F

IC2171



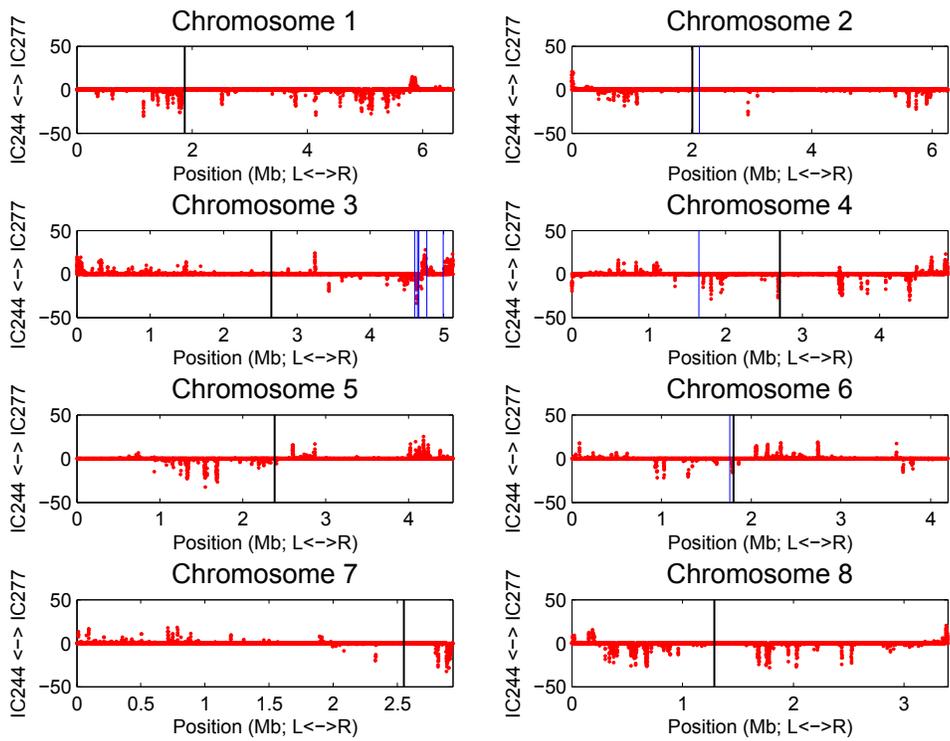
G

IC2205



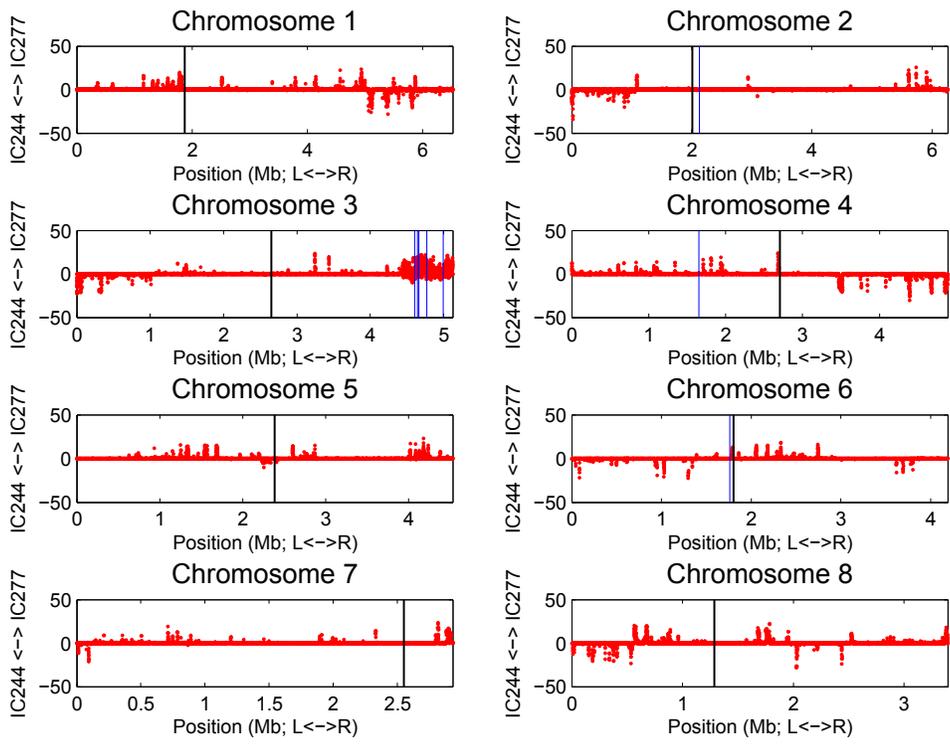
H

IC2207



I

IC2209



CHAPTER 3

ENHANCED DIVERSITY AND AFLATOXIGENICITY IN INTERSPECIFIC HYBRIDS OF *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS*

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Abstract

Aspergillus flavus and *A. parasiticus* are two of the most important aflatoxin-producing species that contaminate agricultural commodities worldwide. Both species are heterothallic and undergo sexual reproduction in laboratory crosses. Here, we examine the possibility of interspecific matings between *A. flavus* and *A. parasiticus*. These species can be distinguished morphologically and genetically, as well as by their mycotoxin profiles.

Aspergillus flavus produces B aflatoxins and cyclopiazonic acid (CPA), B aflatoxins or CPA alone, or neither mycotoxin; *A. parasiticus* produces B and G aflatoxins or the aflatoxin precursor *O*-methylsterigmatocystin, but not CPA. Only four out of forty-five attempted interspecific crosses between compatible mating types of *A. flavus* and *A. parasiticus* were fertile and produced viable ascospores. Single ascospore strains from each cross were isolated and shown to be recombinant hybrids using array comparative genome hybridization (aCGH) and multilocus sequence typing (MLST). Higher total aflatoxin concentrations in the F1 progeny strains compared to midpoint parent aflatoxin levels indicate synergism in aflatoxin production. Three progeny strains synthesized G aflatoxins that were not produced by the parents, which suggest hybridization may be an important diversifying force resulting in the genesis of novel toxin profiles. The genetic clustering of experimental hybrids with naturally occurring *A. flavus* S strains and *A. nomius* based on aCGH and MLST suggests that hybridization may drive genetic and functional diversity in these agriculturally important species.

Introduction

Aflatoxins, of which B₁ is considered the most toxic animal carcinogen (Squire 1981), are secondary metabolites synthesized by several species in *Aspergillus* section *Flavi* (Horn 2007). *Aspergillus flavus* and *A. parasiticus* are responsible for the majority of aflatoxin (AF) contamination events in agronomically important commodities, especially oil-rich crops, worldwide (Horn 2005). Poisoning from ingestion of aflatoxin-contaminated food has resulted in hundreds of documented human and animal deaths worldwide (Giesecker *et al.* 2004; Krishnamachari *et al.* 1975; Probst *et al.* 2007) and is associated with other ailments such as liver cancer and growth stunting in children (Khlanguiset *et al.* 2011). Understanding the genetic and evolutionary mechanisms that increase the aflatoxin-producing potential of these fungi is important as this may guide the development of novel and more cost-effective management strategies to reduce aflatoxin contamination of crops (Wu *et al.* 2013).

Natural populations of *A. flavus* show a high level of variation in mycotoxin production, with individuals producing both AFs and cyclopiazonic acid (CPA), AFs alone, CPA alone or neither mycotoxin (Horn & Dorner 1999; Horn *et al.* 1996). *A. parasiticus* produces B and G AFs or the precursor in the AF biosynthesis pathway, *O*-methylsterigmatocystin (OMST), but not CPA. *A. flavus* and related species are further characterized by two morphotypes: the L strain with large sclerotia >400 µm in diameter and the S strain with abundant small sclerotia <400 µm (Cotty 1989). Although nontoxigenic isolates are fairly common in *A. flavus* L strain (Chang *et al.* 2005; Horn & Dorner 1999) and can predominate in certain fields (Ehrlich 2008), they are rare in *A. flavus* S strain and *A.*

parasiticus (Horn & Dorner 1999). *A. flavus* L strains typically produce B AFs. The S strain of *A. flavus* also produces B AFs (S_B), whereas S strains represented by other species produce either B or B+G AFs (S_{BG}) (Horn 2005). The S_{BG} producers include *A. minisclerotigenes* (Pildain *et al.* 2008), *A. nomius* (Ehrlich *et al.* 2007a) and *A. parvisclerotigenus* (Frisvad *et al.* 2005). Sexual reproduction has been reported in *A. flavus* L strain, *A. parasiticus* and *A. nomius* and is likely responsible for the high genetic variation observed in populations (Horn *et al.* 2009a, 2011; Horn *et al.* 2009b, c). Analysis of DNA sequence variation across multiple intergenic regions in the aflatoxin gene clusters of *A. flavus*, *A. parasiticus* and *A. minisclerotigenes* revealed significant linkage disequilibrium organized into distinct blocks that are conserved across different localities, suggesting that genetic recombination is nonrandom and widespread in these fungi (Moore *et al.* 2013).

The genetic characterization of progeny strains from intraspecific *A. flavus* crosses revealed independent assortment of chromosomes as well as crossovers in the aflatoxin gene cluster (OlarTE *et al.* 2012). In one instance, crossovers in the gene cluster restored aflatoxin production in the nonaflatoxigenic parent. The implication is that loss of function mutations, such as the nonsense mutation in *pksA* (*aflC*) that results in a the complete loss of aflatoxigenicity (Ehrlich & Cotty 2004) or a deletion in *cypA* that knocks out G aflatoxin formation in *A. flavus* (Ehrlich *et al.* 2004), can be rescued via crossovers and independent assortment during sex. Depending on the diversity of toxin production in the parental strains, it is plausible that progeny strains can show both qualitative and quantitative differences in toxin production. Because *A. flavus* and *A. parasiticus* are heterothallic (Horn *et al.* 2009a; Horn *et al.* 2009b, c; Olarte *et al.* 2012; Ramirez-Prado *et al.* 2008) and show extensive niche

overlap (Moore *et al.* 2013) and DNA relatedness (Kurtzman *et al.* 1986), successful inter-specific mating could potentially create progeny strains with novel mycotoxin combinations. Herein we examine the outcomes of hybridization between *A. flavus* and *A. parasiticus* in laboratory crosses and compare the genetic composition of hybrid progeny strains to naturally occurring aflatoxigenic fungi.

Materials and Methods

Interspecific crosses

Aspergillus flavus and *A. parasiticus* were sampled from soil and peanut seeds in peanut fields from Terrell County, Georgia, USA, with the exception of IC314 (peanut field soil, Bladen County, NC), IC311 (peanut field soil, Conecuh County, AL), IC309 (peanut field soil, Covington County, MS) and AF36 (cottonseed, Yuma Valley, AZ). Isolates were characterized according to morphology, vegetative compatibility and aflatoxin production (Horn & Dorner 1999; Horn & Greene 1995; Horn *et al.* 1996) as well as mating type (Ramirez-Prado *et al.* 2008) and multilocus aflatoxin cluster haplotype (Carbone *et al.* 2007). Forty-five crosses between *A. flavus* and *A. parasiticus* strains of the opposite mating type were set up to examine the possibility of hybridization between the two species (see Table 3.1). Fungal strains were mated in the laboratory as described previously (Horn *et al.* 2009b). Briefly, strains were grown on Czapek agar (CZ) slants for 14 days at 30 °C, conidia from strains of the opposite mating type were suspended in dilute water agar, and conidial suspensions were each spread onto five slants of mixed cereal agar. Slants were incubated in darkness for 14 days at 30 °C to induce sclerotium production and subsequently incubated in

Table 3.1. Morphological comparisons between *A. flavus* and *A. parasiticus* parents and their hybrid progeny^a

Strain	Species	Parent/progeny	Colony diam (mm) ^b	Sclerotial weight (g) ^c	Stipe length (μm) ^d	Conidial diam (μm) ^e	Conidial head seriation ^f
IC278	<i>A. flavus</i>	<i>MATI-1</i> parent	41 ± 1.5 a	65.4 ± 2.5 a	495.5 ± 135.1 a	4.1 ± 0.54 a	33% uniseriate/67% biseriate
IC327	<i>A. parasiticus</i>	<i>MATI-2</i> parent	36 ± 1.0 b	26.0 ± 2.2 b	248.4 ± 78.4 b	5.0 ± 0.45 b	99% uniseriate/1% biseriate
IC1603	Hybrid	Progeny	31 ± 1.1 c	0.5 ± 0.5 c	106.1 ± 32.4 c	4.0 ± 0.45 a	100% uniseriate
IC278	<i>A. flavus</i>	<i>MATI-1</i> parent	41 ± 1.5 a	65.4 ± 2.5 a	495.5 ± 135.1 a	4.1 ± 0.54 a	33% uniseriate/67% biseriate
IC33	<i>A. parasiticus</i>	<i>MATI-2</i> parent	34 ± 0.9 b	32.1 ± 9.1 b	132.5 ± 44.0 b	5.4 ± 0.44 b	100% uniseriate
IC1637	Hybrid	Progeny	36 ± 1.2 c	0.0 ± 0.0 c	266.1 ± 90.6 c	4.2 ± 0.37 a	98% uniseriate/2% biseriate
IC278	<i>A. flavus</i>	<i>MATI-1</i> parent	41 ± 1.5 a	65.4 ± 2.5 a	495.5 ± 135.1 a	4.1 ± 0.54 a	33% uniseriate/67% biseriate
IC65	<i>A. parasiticus</i>	<i>MATI-2</i> parent	36 ± 0.8 b	25.1 ± 2.1 b	111.9 ± 21.8 b	4.7 ± 0.41 b	100% uniseriate
IC1612	Hybrid	Progeny	43 ± 1.2 c	1.9 ± 1.9 c	517.6 ± 166.3 a	4.4 ± 0.60 a	99% uniseriate/1% biseriate
IC1616	Hybrid	Progeny	32 ± 1.0 d	0.2 ± 0.3 c	105.5 ± 37.6 b	5.4 ± 0.50 c	99% uniseriate/1% biseriate
IC278	<i>A. flavus</i>	<i>MATI-1</i> parent	41 ± 1.5 a	65.4 ± 2.5 a	495.5 ± 135.1 a	4.1 ± 0.54 ab	33% uniseriate/67% biseriate
IC324	<i>A. parasiticus</i>	<i>MATI-2</i> parent	36 ± 1.2 b	38.7 ± 1.2 b	96.5 ± 25.2 b	5.0 ± 0.39 c	100% uniseriate
IC1622	Hybrid	Progeny	35 ± 1.1 b	33.8 ± 1.3 b	240.8 ± 86.6 c	3.9 ± 0.39 a	88% uniseriate/12% biseriate
IC1630	Hybrid	Progeny	30 ± 1.1 c	32.7 ± 4.0 b	88.3 ± 33.5 b	4.3 ± 0.45 b	100% uniseriate

^aMeans ± SD followed by a different letter within a parent-progeny group are significantly different (P<0.05).

^bMeans based on 12 colonies on CZ plates (7 d, 25°C).

^cMeans based on three CZ slants (14 d, 30°C).

^dMeans based on 20 stipes from CZ plates (7 d, 25°C).

^eMeans based on 30 conidia from CZ slants (14 d, 30°C).

^fSeriations based on 100 conidial heads from CZ plates (7 d, 25°C).

sealed plastic bags for an additional 6–9 months at 30 °C. For each interspecific cross, at least 300 sclerotia, when available, were sliced open and examined for ascocarps and ascospores. Single ascospore progeny isolates were obtained from individual ascocarps within stromata, as described previously (Horn *et al.* 2009b).

Light microscopy and SEM

Parental strains of *A. flavus* and *A. parasiticus* and their F1 hybrid progeny were compared morphologically for colony diameter, sclerotia production, stipe length and conidial head seriation and for the diameter, ornamentation and color (when en masse) of conidia. Six progeny strains (IC1603, IC1612, IC1616, IC1622, IC1630 and IC1637) showing qualitative or quantitative differences in toxin profiles compared to parental strains were examined (Table 3.1). Strains were grown on CZ slants in darkness for 14 d at 30 C for obtaining sclerotia and for measuring conidial diameter and assessing conidial ornamentation with the light microscope (Leica DM2500). Sclerotia were harvested and cleaned from three slants per strain according to Horn *et al.* (2009b) and dried over desiccant for two weeks before weighing. Three-point inoculated CZ plates were incubated in darkness at 25 C for 7 d for measuring colony diameter (four plates per strain), stipe length and conidial head seriation. Those plates were further incubated at 25 C (14 d) for determining the color of conidia en masse on colonies. Conidial color was based on Kornerup & Wanscher (1978). Morphological measurements were compared statistically with a one-way ANOVA followed by Tukey's test for multiple comparisons of means.

Stromata from two fertile *A. flavus* × *A. parasiticus* crosses (IC278 × IC324 and IC278 × IC327) that were among crosses with the highest number of ascospore-bearing ascocarps (Table 1) were dissected and imaged using both light microscopy and scanning electron microscopy (SEM). Hand-sectioned stromata were examined using both a stereomicroscope and compound microscope. For SEM imaging, samples were fixed using gluteraldehyde/sodium cacodylate buffer and then critical-point dried, as described previously (Horn *et al.* 2009b).

Mycotoxin quantification and heritability

Vials (4 mL) containing 1 mL of yeast-extract sucrose broth were inoculated with dry conidia ($\sim 1 \times 10^5$) to determine AF, OMST, and CPA production by parents and F1 hybrids (Horn & Dorner 1999). Cultures were maintained undisturbed in darkness for 7 days at 30°C.

Production of AF, OMST and CPA in vial cultures was analyzed by high performance liquid chromatography as previously described (Horn & Dorner 1999; Horn *et al.* 1996).

Quantification limits were 0.5 ng of AF B₁ and 2 µg of CPA per mL of culture medium.

Previously we determined that twelve offspring in each of nine different *A. flavus* crosses were sufficient for estimates of total AF (B₁, + B₂) and CPA heritability (Olarde *et al.* 2012).

A similar experimental design was followed in the present study with 11-12 progeny examined for each of four *A. flavus* × *A. parasiticus* interspecific crosses and 17-30 progeny from five *A. parasiticus* × *A. parasiticus* intraspecific crosses. Each individual progeny strain was assayed after seven days of growth in culture vials inoculated with conidia from three points (biological replicates) within the culture slant (top, middle and bottom). For

each strain, we determined the total AF ($B_1 + B_2 + G_1 + G_2$) and CPA concentrations and these were then averaged across the three replicates to obtain final AF and CPA concentrations for each strain.

Total AF and CPA concentrations of offspring were plotted against mid-parent values for each cross, and mycotoxin heritability was estimated as the slope of the resulting best-fit regression line (OlarTE *et al.* 2012). Heritability of AF and CPA in interspecific crosses was plotted with corresponding heritability estimates in *A. flavus* and *A. parasiticus* intraspecific crosses. To determine whether mycotoxin production in the hybrid offspring of each interspecific cross was significantly higher than in the parental species, we first tested for a significant difference in mean total AF and CPA concentrations between progeny and parental isolates using ANOVA followed by Tukey's multiple comparisons test implemented in the R statistical software (R Development Core Team 2010). All tests were based on three biological replicates for each parental and progeny strain.

Aflatoxin concentration curves

A time course experiment over 3, 5 and 7 days was performed to evaluate aflatoxin production in liquid culture for four interspecific parent-offspring trios (IC278, IC327, IC1602; IC278, IC65, IC1612; IC278, IC324, IC1622; and IC278, IC33, IC1643) showing significantly different AF concentrations. Fifty-mL conical Falcon tubes (Corning) containing 20 mL of YES broth were each inoculated with 1 mL of conidial suspension ($\sim 5 \times 10^6$ conidia/mL). Tubes were incubated with shaking at 30°C. At the end of each time point, 10 mL of chloroform was added directly to each culture tube and AFs were extracted

with additional shaking overnight (~15 hours). The chloroform layer was transferred to a new 50 mL Falcon tube and allowed to evaporate, at which point 1 mL methanol was added; samples were then stored at -80 °C. This process was repeated two more times with an extraction period of two hours.

Aflatoxins B₁, B₂, G₁, G₂ were separated with high performance liquid chromatography/mass spectrometry (2010EV LC/PDA, Shimadzu, Japan) using an Eclipse XDB-C18 analytical column (250 mm x 4.6 mm, 5 µm, Agilent, Santa Clara, CA). The mobile phase solvents consisted of 1% acetic acid in water (solvent A; HPLC grade acetic acid and LC-MS grade water) and 100% acetonitrile (solvent B; LC-MS grade). To separate the different aflatoxins, a gradient solvent system with the following ratios of solvent A to B was used: 90:10 (0-5 min); 90:10 to 88:12 (5-10 min); 88:12 to 80:20 (10-20 min); 80:20 to 75:25 (20-30 min); 75:25 to 65:35 (30-35 min); 65:35 to 60:40 (35-40 min); 60:40 to 50:50 (40-55 min); and 50:50 to 10:90 (55-60 min). The column was then washed for 10 min with 10% solvent B. The flow rate was 0.4 mL/min and the injection volume was 10 µL. The UV spectrum was recorded from 190 to 800 nm. Chromatographic profiles of aflatoxins B₁, B₂, G₁, G₂ were recorded and analyzed at 280 nm and 340 nm, respectively.

Ploidy

Aspergillus flavus and *A. parasiticus* have been reported to have multinucleate conidia (Yuill 1950) and can be haploid or diploid and homokaryotic or heterokaryotic (Horn *et al.* 2011; Maruyama *et al.* 2001; Papa 1973, 1978). Because haploid and diploid conidial diameters do not differ significantly (Papa 1973, 1978), flow cytometry was performed to detect ploidy

changes in the putative hybrid progeny compared to parental strains. Fungi were grown on CZ plates at 30 °C in darkness for 5 days. Conidia were obtained by flooding plates with 500 µL Tween-saline solution. One mL of the conidial suspension was filtered through a single layer Miracloth (Calbiochem, USA) into a 2 mL tube and 1 mL 70% ethanol was added, followed by incubation at 4°C for 30 minutes. Conidia were concentrated by centrifugation at 3000 rpm for 5 min, washed twice with Tween-saline solution, resuspended in 500 µL of TE buffer, and incubated 1 h at 37°C with RNase (1mg/mL). For DNA staining, propidium iodide (25 µg/mL) was added to the conidial suspension and incubated at room temperature for 30 min. Conidial density was adjusted to $\sim 10^7$ /mL and sorted on a flow cytometer (Becton–Dickinson FACScan) using fluorescence-activated cell sorting (FACS). Data acquisition and fluorescence analysis to determine the number of nuclei in each conidium were performed using the Cell Quest Pro software (BD Biosciences, San Jose CA). The formation of multiple peaks in fluorescence histograms was interpreted as differences in conidial DNA content such that the presence of two sharp peaks corresponded to conidial populations that were either haploid and monokaryotic (1N), dikaryotic (1N + 1N) or diploid (2N). Reference haploid *A. flavus* NRRL 19060, NRRL A-27460 and diploid NRRL 19301, 649×86 (Woloshuk *et al.* 1995) strains were used as standards in FACS. In the fluorescence histograms of diploids, the two peaks were shifted to the right such that the first peak corresponded to conidia that were mostly monokaryotic (2N) and the second peak to dikaryotic (2N + 2N) conidia.

DNA extraction and multilocus sequencing

Lyophilized mycelial pellets were pulverized using a Retsch MM301 tissue homogenizer (Retsch, Inc., Newtown, PA) and total genomic DNA was extracted using either the Qiagen maxiprep kit (Qiagen, Valencia, CA) or the MasterPure Yeast DNA Purification Kit (Epicentre Technologies, Madison, WI). PCR amplification and DNA sequencing for multilocus sequence typing (MLST) were as described previously (Moore *et al.* 2009; Carbone *et al.* 2007). For each locus, DNA sequences were aligned, visually inspected and edited using Sequencher Version 4.7 (Gene Codes Corporation, Ann Arbor, MI).

Alignments were exported as NEXUS files and analyzed using Mobylye SNAP Workbench (Monacell & Carbone 2014; Price & Carbone 2005). Multilocus alignments were generated using SNAP Combine (Aylor *et al.* 2006) and clone-corrected by collapsing into haplotypes using SNAP Map (Aylor *et al.* 2006). Collapsing was performed with the option of recoding insertions/deletions (indels) for maximal haplotype resolution.

Recombination

Genome-wide recombination events arising from independent assortment of chromosomes were detected by examining variation segregating at four loci that span three chromosomes: *aflC/aflD*, *aflM/aflN* and *aflW/aflX* located in the aflatoxin gene cluster on chromosome 3, the mating type (*MAT*) gene on chromosome 6, and tryptophan synthase (*trpC*) on chromosome 4 (Table 3.2). For each locus, the null hypothesis of no significant difference in the frequency of parental alleles in the progeny sampled from each cross was tested using a binomial test implemented in MS Excel. In crosses that produce progeny with no crossovers

Table 3.2. Incidences of sexual state in forty-five attempted interspecific crosses between compatible mating types of *Aspergillus flavus* and *A. parasiticus*

<i>MAT 1-1¹</i>			<i>MAT 1-2¹</i>			Number of sclerotia/stromata per slant ⁴	Number of sclerotia/stromata examined	% with ascocarps ^{4,5}	% with ascospore- bearing ascocarps ^{4,6}
IC strain	NRRL strain ²	VCG ³	IC strain	NRRL strain ²	VCG ³				
<i>A. flavus</i> × <i>A. parasiticus</i>									
IC289	29518	44	IC69	29606	10	0	-	-	-
IC314	50428	79	IC65	29602	8	24 ± 17	113	0	-
IC314	50428	79	IC33	29570	3	47 ± 21	237	0	-
IC283	29512	38	IC67	29604	9	47 ± 30	234	0	-
IC289	29518	44	IC324	35779	-	83 ± 37	376	0	-
IC314	50428	79	IC324	35779	-	121 ± 49	309	0	-
IC308	29537	63	IC59	29596	6	398 ± 63	300	0	-
IC283	29512	38	IC65	29602	8	623 ± 114	300	0	-
IC289	29518	44	IC65	29602	8	627 ± 196	300	0	-

Table 3.2. Continued

IC283	29512	38	IC324	35779	-	669 ± 50	300	0	-
IC283	29512	38	IC329	35784	-	710 ± 6	300	0	-
IC289	29518	44	IC330	35785	-	221 ± 46	300	0.7 ± 1.1	0
IC308	29537	63	IC65	29602	8	418 ± 30	300	0.7 ± 1.1	0
IC311	35737	-	IC331	35786	-	1650 ± 101	300	2.7 ± 1.1	0
IC311	35737	-	IC324	35779	-	1225 ± 11	300	6.3 ± 3.2	0
IC311	35737	-	IC65	29602	8	1053 ± 36	300	8.7 ± 4.0	0
IC311	35737	-	IC72	29609	13	613 ± 68	300	12.7 ± 0.6	0
IC308	29537	63	IC324	35779	-	891 ± 42	300	0.3 ± 0.6	0.3 ± 0.6
IC308	29537	63	IC328	35783	-	635 ± 14	300	1.0 ± 1.0	0.7 ± 0.6
IC278	29507	33	IC33	29570	3	232 ± 66	300	12.3 ± 5.0	6.0 ± 2.6
IC278	29507	33	IC327	35782	-	324 ± 32	420	28.4 ± 9.3	10.6 ± 3.9
IC278	29507	33	IC324	35779	-	398 ± 59	400	36.3 ± 9.2	26.7 ± 6.5
IC278	29507	33	IC65	29602	8	282 ± 133	300	49.7 ± 7.0	33.7 ± 5.9

Table 3.2. Continued

<i>A. parasiticus</i> × <i>A. flavus</i>									
IC61	29598	7	IC1179	AF36	YV36	4 ± 8	20	0	-
IC1	29538	1	IC1179	AF36	YV36	8 ± 4	40	0	-
IC73	29610	14	IC1179	AF36	YV36	23 ± 12	115	0	-
IC49	29586	4	IC1179	AF36	YV36	33 ± 14	164	0	-
IC65	29602	8	IC271	29500	28	37 ± 14	147	0	-
IC49	29586	4	IC229	29459	6	62 ± 42	283	0	-
IC65	29602	8	IC309	35735	-	71 ± 57	289	0	-
IC65	29602	8	IC1179	AF36	YV36	126 ± 26	306	0	-
IC73	29610	14	IC201	21882	24	674 ± 98	300	0	-
IC65	29602	8	IC201	21882	24	1184 ± 351	300	0	-
IC65	29602	8	IC277	29506	32	1430 ± 96	300	0	-
IC74	29611	15	IC201	21882	24	736 ± 49	300	0.3 ± 0.6	0
IC65	29602	8	IC229	29459	6	484 ± 142	300	1.0 ± 1.7	0

Table 3.2. Continued

IC75	29612	16	IC229	29459	6	18 ± 9	90	1.1 ± 2.5	0
IC73	29610	14	IC229	29459	6	186 ± 111	300	1.3 ± 0.6	0
IC49	29586	4	IC201	21882	24	689 ± 87	300	2.0 ± 1.7	0
IC53	29590	5	IC229	29459	6	12 ± 7	58	2.0 ± 4.5	0
IC44	29581	4	IC201	21882	24	677 ± 129	300	3.0 ± 2.0	0
IC73	29610	14	IC202	29506	32	734 ± 40	300	4.0 ± 3.6	0
IC18	29555	2	IC202	29506	32	610 ± 114	300	7.7 ± 4.2	0
IC49	29586	4	IC277	29506	32	806 ± 40	300	12.3 ± 3.5	0
IC70	29607	11	IC202	29506	32	632 ± 148	300	19.0 ± 3.6	0

¹Mating-type designations IC278, IC33 and IC327 are from Ramirez-Prado *et al.* (2008).

²Strain numbers (NRRL) from Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

³Vegetative compatibility groups based on Horn and Greene (1995) and Horn and Dorner (1999) except for YV36 (Ehrlich *et al.* 2007b). Strains of unknown VCG are indicated with a ‘-’.

⁴Means ± s.d. (*n* = 3-5 culture slants or biological replicates).

Table 3.2. Continued

⁵Percentage of total number of sclerotia/stromata examined containing one or more ascocarps irrespective of the presence of ascospores.

⁶Percentage of total number of sclerotia/stromata examined containing one or more ascospore-bearing ascocarps.

in the AF cluster, we would expect Mendelian segregation of loci on different chromosomes to result in only $(0.5)^3$ or 13% of the progeny sharing a parental MLST. Within each cross, significant differences in toxin production between hybrid offspring and parental isolates were interpreted within the context of MLSTs based on five loci (*aflC/aflD*, *aflM/aflN*, *aflW/aflX*, *MAT*, and *trpC*) spanning three linkage groups. Specifically we examined whether progeny strains showed evidence of independent assortment, and whether progeny individuals that shared a multilocus haplotype had significant differences in AF and CPA production.

We examined a cluster-specific MLST for reconstructing crossover events in the AF gene cluster. This was based on three intergenic regions in the AF cluster (*aflC/aflD*, *aflM/aflN* and *aflW/aflX*) that flank hot and cold spots of recombination in *A. flavus* (Moore *et al.* 2009) and *A. parasiticus* (Carbone *et al.* 2007). A crossover between these loci in the parents would result in progeny genotypes with a different combination of parental alleles.

Array comparative genome hybridization (aCGH)

Hybrid progeny showing unique mycotoxin phenotypes and cluster MLSTs compared to their parents were further genotyped using aCGH. Parent-offspring trios were examined to identify putative crossovers within the aflatoxin gene cluster and to identify genome-wide hybridization events between *A. flavus* and *A. parasiticus*. For each trio strain, total genomic DNA was isolated and labeled with the BioPrime DNA labeling System (Invitrogen Catalogue No. 18094-011), followed by hybridization to whole genome *A. flavus* NRRL 3357 Affymetrix GeneChip microarrays, as previously described (Olarie *et al.* 2012). A total

of fifty-six isolates representing variation across intra- and inter-specific crosses, as well as isolates sampled from populations of *A. flavus* and *A. parasiticus* and isolates of closely related species in *Aspergillus* section *Flavi*, were subjected to aCGH (Table S3.1).

Microarray slides were hybridized, scanned and analyzed by Expression Analysis (Raleigh-Durham, NC).

Genome-wide dendrogram and aflatoxin cluster heat maps

Microarray probes or probe sets that hybridized more completely with labeled DNA had a higher sequence similarity to the reference *A. flavus* NRRL 3357 genome sequence. To quantify the degree of similarity and to identify putative groups of very similar strains and species, microarray data were imported into JMP genomics (SAS, Cary, NC, USA) for further transformation and normalization, as previously described (OlarTE *et al.* 2012).

Briefly, data were log₂ transformed, normalized using Loess normalization with no background correction, and exported in MS Excel format. We evaluated parental inheritance in the offspring and identified putative recombination breakpoints by comparing hybridization intensities for each of six parent-offspring trios, as described previously (OlarTE *et al.* 2012). A heat map showing the degree of DNA sequence variation in telomeric and subtelomeric regions of chromosome 3R, which span the CPA and AF gene clusters, was generated using the *imagesc* function in Matlab (MathWorks Inc., Natick, MA). A similarity dendrogram was generated in JMP genomics based on clustering using Pearson product-moment correlations (Hane *et al.* 1993) to identify groups of genetically similar strains and species.

Genome-wide parentage plots

Patterns of inheritance in the six parent-offspring trios for all eight chromosomes were examined to detect crossovers and evidence of independent assortment in putative hybrids. In the parentage plots, probe data were normalized using Loess normalization but not log₂ transformed, as described previously (OlarTE *et al.* 2012). The plots for each parent-offspring trio show the approximate physical location of each probe on the chromosome and the degree of similarity of the offspring to each parent on the y-axis. For example, a positive difference translates to an offspring showing more similarity to the top parent (*A. flavus*) in the plot while a negative difference suggests that the offspring is more similar to the bottom parent (*A. parasiticus*). MLST loci that uniquely distinguish parents and offspring were used to validate patterns of inheritance in parentage plots.

Principal component analysis and phylogenetic inference

The genetic relatedness of hybrid progeny to naturally occurring aflatoxigenic species was examined using principal component analysis (PCA) and phylogenetic reconstruction of MLST loci. Previously we examined variation in *MAT*, *aflM/aflN*, *aflW/aflX* and *trpC* for 758 isolates of *A. flavus*, *A. parasiticus* and *A. minisclerotigenes* sampled from single peanut fields in the United States (Georgia), Africa (Benin), Argentina (Córdoba), Australia (Queensland) and India (Karnataka) (Moore *et al.* 2013). Variation in the expanded sample of experimental and natural isolates was first examined to determine the number of significant axes of variation using the Tracy-Widom statistic (Tracy & Widom 1994). The optimal number of distinct clusters that associated with subpopulations or species was based

on the Gap Statistic, which compares within cluster variance with that expected under a reference null distribution based on PCA (Tibshirani *et al.* 2001). The association of statistically significant clusters with geographic locality and species was displayed graphically using 3D scatter plots, generated using the SCATTERPLOT3D package in R (Ligges & Mächler 2003). In the scatterplot, each individual is a single point in three-dimensional space and the species and sampling locality of each individual is also plotted as a different color and symbol. All methods used in this analysis are implemented in Mobylye SNAP Workbench, a web-based analysis portal deployed at North Carolina State University (Monacell & Carbone 2014).

To determine the ancestral relationships among clades inferred from PCA analysis, we subjected the four-locus MLST data to maximum likelihood analysis with rapid bootstrapping using RAxML (Stamatakis *et al.* 2008). We performed maximum likelihood bootstrap analysis by specifying distinct partitions for each locus comprising the MLST and assuming different rates of nucleotide substitutions.

Results

Interspecific crosses

Crosses between *A. flavus* and *A. parasiticus* showed considerable variation in the number of sclerotia/stromata per slant, the proportion of sclerotia/stromata with ascocarps, and the proportion of sclerotia/stromata containing ascospore-bearing ascocarps (Table 3.2). Of the forty-five attempted interspecific crosses, twenty-three produced ascocarps. The majority of ascocarps were small and sometimes hollow and showed no evidence of ascogenous hyphae

or asci (Fig. 3.1B). Six of these crosses produced ascospore-bearing ascocarps of which four crosses (IC278 × IC33, IC278 × IC327, IC278 × IC324 and IC278 × IC65) produced viable ascospores and ultimately progeny. The majority of ascospores within these ascocarps appeared collapsed and empty; refractive and possibly viable ascospores were rarely observed (<1 in a 1000) (Fig. 3.1G). Progeny were obtained only from crosses between *A. flavus* IC278 (*MATI-1*) and four *A. parasiticus* strains (*MATI-2*); none of the crosses between *A. parasiticus* (*MATI-1*) and *A. flavus* (*MATI-2*) produced ascospores (Table 3.2). The number of sclerotia produced did not correlate with the incidence of ascospore-bearing ascocarps, as seen in cross IC278 × IC65, which had the highest fertility (33.7% of sclerotia/stromata) but was one of the lower sclerotial producers.

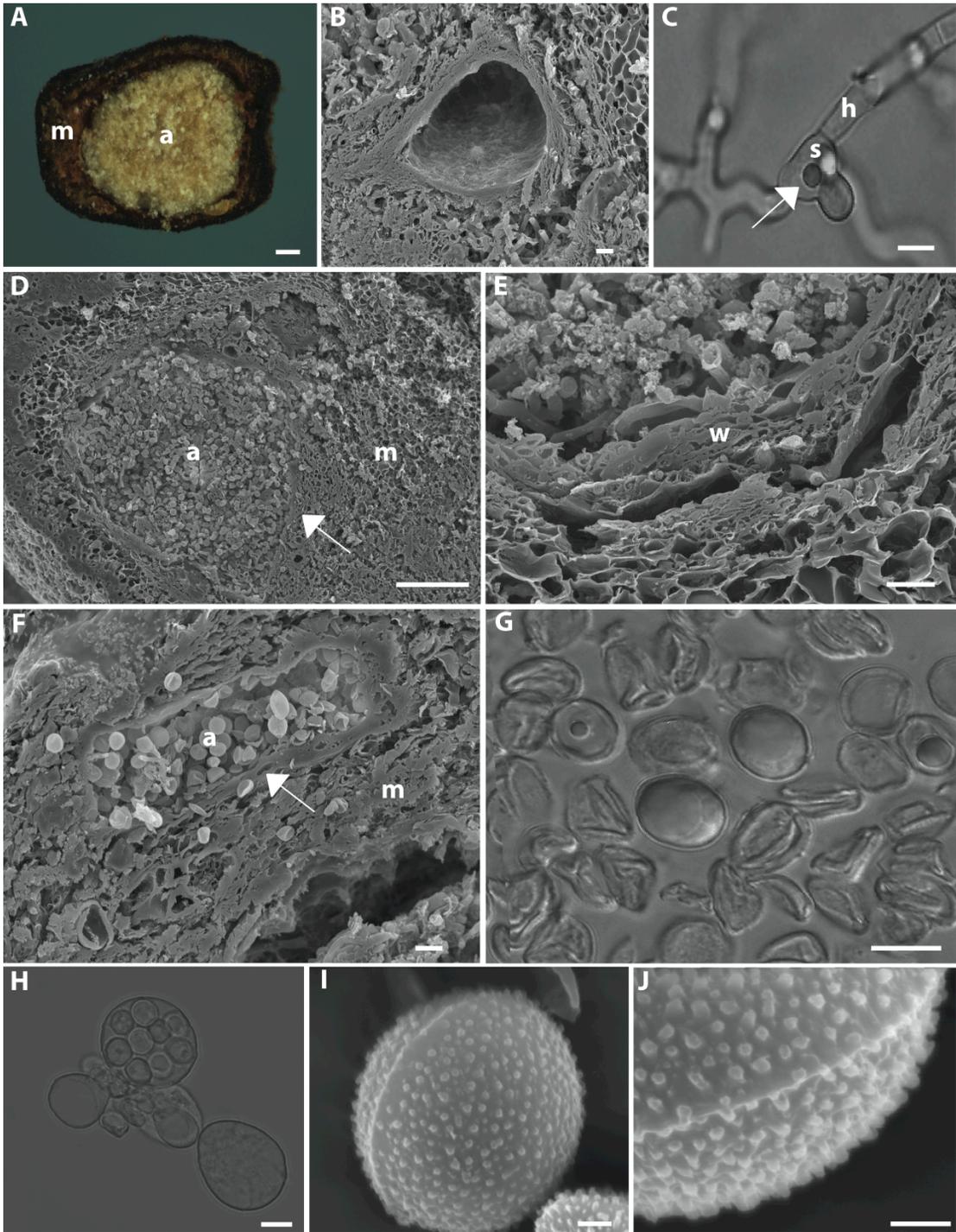
Light microscopy and SEM

In the four interspecific crosses that produced progeny, parental *A. flavus* IC278 showed significantly greater growth rate, longer stipes and smaller conidia compared to the four parental *A. parasiticus* strains (Table 3.1), which is consistent with described morphological differences between the two species (Klich & Pitt 1988; Raper & Fennell 1965). Also consistent with described species differences, *A. flavus* conidial heads were predominantly biseriate (67%), conidial color was grayish green (29E5; Kornerup & Wanscher 1978) and conidia were finely roughened, whereas *A. parasiticus* conidial heads were uniseriate (99-100%), conidial color was dark green (27F6) and conidia were more coarsely roughened. Sclerotia production in *A. flavus* NRRL IC278 was significantly greater than production by the four parental *A. parasiticus* strains (Table 3.1).

Progeny strains from interspecific crosses showed a combination of morphological characters that were *A. flavus*-like, *A. parasiticus*-like or intermediate between the two species (Table 3.1). Conidia in progeny strains resembled those of parental *A. flavus* IC278 in size and ornamentation (nearly smooth to finely roughened); progeny strain IC1616 was an exception in having conidia significantly larger than either parent. In contrast, the predominantly uniseriate conidial heads in all progeny (88-100%) were more suggestive of *A. parasiticus*. Conidial color was typically intermediate in hue and/or tone between the two species (28F6, 28E-F7, 29E-F5). Stipe length in progeny was more strain specific, with stipes being relatively short as in *A. parasiticus* (IC1603, IC1616, IC1630), intermediate in length between the two species (IC1637, IC1622), or relatively long as in *A. flavus* (IC1612) (Table 3.1). Sclerotium production in progeny was significantly less than either parent, with the exception of the two progeny strains from *A. flavus* IC278 × *A. parasiticus* IC324 in which sclerotium production was similar to *A. parasiticus*. One progeny strain (IC1630) was morphologically unstable and showed sectoring and scattered *A. flavus*-like conidial heads.

Interspecific matings between *A. flavus* and *A. parasiticus* produced indehiscent ascocarps within the matrix of stromata (Figs. 3.1A, D, F). Ascocarp size was $184 \pm 69.8 \times 143 \pm 50.2 \mu\text{m}$ (\pm SD; n = 41) in infertile ascocarps and $483 \pm 127.4 \times 404 \pm 119.8 \mu\text{m}$ (n = 36) in ascospore-bearing ascocarps. An ascocarp peridium separated the contents from the stromal matrix (Figs. 3.1D, F). Asci formed within ascocarps and were $22.7 \pm 2.17 \times 20.9 \pm 2.18 \mu\text{m}$ (n = 27) (Fig. 1H); each ascus typically contained eight ascospores but other numbers of ascospores were not uncommon. Ascospores were oblate and globose to subglobose in face view ($10.4 \pm 1.34 \times 9.7 \pm 1.24 \mu\text{m}$; n = 50), contained a single oil droplet,

Figure 3.1. Sexual reproductive structures in hybrid crosses between *Aspergillus flavus* and *A. parasiticus*. A: Sectioned stroma containing one ascospore-bearing ascocarp; B: SEM image of aborted ascocarp; C: Germinated ascospore; arrow shows oil droplet outside of the spore; D: SEM image of stroma in cross section with one ascospore-bearing ascocarp; arrow shows ascocarp peridium; E: SEM image showing structure of ascocarp peridium; F: SEM image of ascocarp-bearing ascospore; arrow shows ascocarp peridium; G: Two viable-appearing ascospores containing oil droplets, surrounded by collapsed empty ascospores; H: Ascus containing nine ascospores; I, J: SEM image of ascospore with finely tuberculate ornamentation and an equatorial ridge. Abbreviations: a, ascocarp; h, hypha; m, stromal matrix; p, ascocarp peridium; s, ascospore. Scale bars: A, D: 100 μm ; B, C, E, H: 10 μm ; I, J: 1 μm .



and were finely roughened and encircled by a distinct equatorial ridge (Figs. 3.1G, I, J).

During ascospore germination, the oil droplet either remained within the spores or migrated a short distance with the germ tube (Fig. 3.1C).

Mycotoxin quantification and heritability

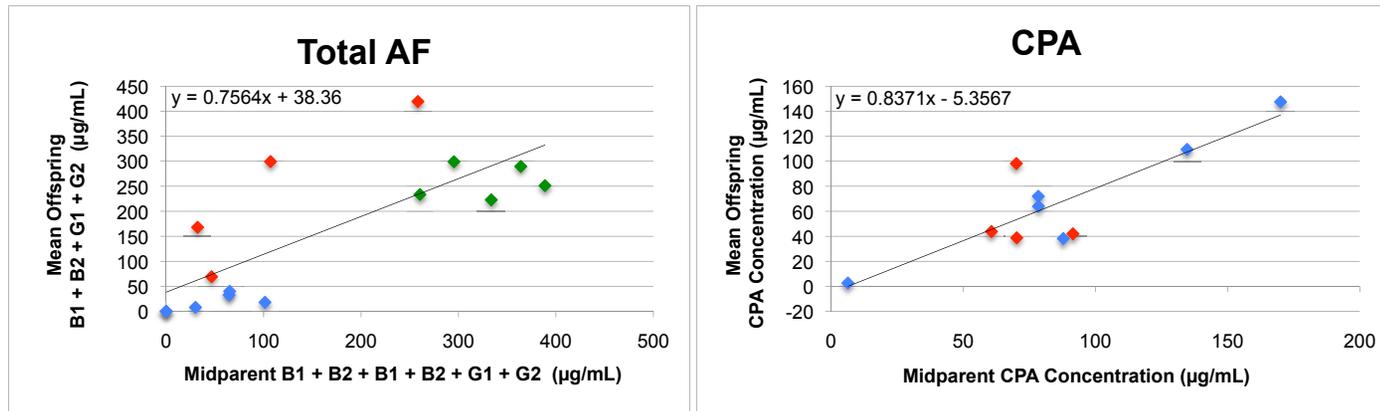
Mean parental and progeny AF B₁, B₂, G₁, G₂, OMST and CPA concentrations ($\mu\text{g/mL}$; n = 3) are shown in Table 3.2. The calculated heritability (h^2) was 0.7564 ± 0.1740 (95% CI: 0.3805, 1.1322) for AF (B₁ + B₂ + G₁ + G₂) and 0.8371 ± 0.1767 (95% CI: 0.4297, 1.2445) for CPA production (Fig. 3.2). Out of a total of forty-eight assayed progeny, there were seventeen in which total AF production was found to be significantly higher than the *A. flavus* parent; ten hybrid progeny in which total AF production was found to be significantly higher than the *A. parasiticus* parent; and seven hybrid progeny were found to produce significantly higher total AF than either parent in the cross (Table 3.3). Three progeny (IC1611, IC1622 and IC1630) synthesized G₁ and G₂ aflatoxins that were not produced by the parents. Fourteen hybrid progeny showed significantly higher CPA production than the *A. flavus* parent.

Aflatoxin concentration curves

Progeny strains consistently showed higher total aflatoxin production than parental strains after 3, 5 and 7 days of growth. With the exception of IC1622, which showed a slight decrease in AF production over the three time points, aflatoxin production increased up to day 7 for all parents and offspring (Fig. 3.3).

Figure 3.2. Total aflatoxin and CPA heritability in intra and interspecific crosses of *A. flavus* and *A. parasiticus*. Blue diamonds denote mean offspring toxin concentration versus midparent toxin concentration for each *A. flavus* × *A. flavus* cross; green diamonds denote mean offspring toxin concentration versus midparent toxin concentration for each *A. parasiticus* × *A. parasiticus* cross; and red diamonds denote mean offspring toxin concentration versus midparent toxin concentration for each hybrid *A. flavus* × *A. parasiticus* cross.

Mycotoxin Heritability



Crosses

A. flavus × *A. flavus*

A. parasiticus × *A. parasiticus*

A. flavus × *A. parasiticus*



Table 3.3. Multilocus genotypes and toxin phenotypes from four *Aspergillus flavus* × *A. parasiticus* crosses

IC Strain	NRRL	Loci examined ^a					OMST ^d (µg/mL)	Aflatoxin					CPA (µg/mL) ^e	Cluster MLST ^f	Genome MLST ^g
		Chr. 6 <i>MAT</i>	Chr. 3 <i>aflC/aflD</i>	Chr. 3 <i>aflM/aflN</i>	Chr. 3 <i>aflW/aflX</i>	Chr. 4 <i>trpC</i>		B ₁ (µg/mL)	B ₂ (µg/mL)	G ₁ (µg/mL)	G ₂ (µg/mL)	Total AFs (µg/mL)			
Parents															
IC278	29507	1	H1	H1	H1	H1	1.4 (0.7)	119.0 (51.1)	3.1 (1.7)	0 (0)	0 (0)	122.1 (52.7)	140.5 (4.2)	H1	H1
IC327	35782	2	H2	H2	H2	H2	0.2 (0.0)	210.9 (42.4)	9.9 (2.1)	169.5 (28.9)	4.2 (0.8)	394.5 (74.1)	0 (0)	H2	H2
Progeny															
Ascocarp 1 ^h															
IC1596	62734	1	H1	H1	H1	H1	0.4 (0.1)	23.1 (12.8)	0.5 (0.3)	0 (0)	0 (0)	23.6 (13.1)**	18.2 (6.9)*	H1	H1
IC1597	62735	2	H1	H1	H1	H2	0.1 (0.1)	20.9 (7.6)	0.3 (0.1)	0 (0)	0 (0)	21.2 (7.8)**	91.3 (39.8)***	H1	H4
Ascocarp 2															
IC1598	62736	1	H2	H2	H2	H2	1.0 (0.4)	127.0 (18.5)	3.1 (0.8)	143.2 (50.8)	2.3 (1.1)	275.6 (62.7)	0 (0)*	H2	H3
IC1599	62737	1	H2	H2	H2	H2	1.4 (0.5)	347.1 (24.4)	10.9 (0.7)	234.4 (7.8)	4.7 (0.1)	597.1 (33.0)*	0 (0)*	H2	H3
IC1600	62738	1	H2	H2	H2	H2	1.7 (0.2)	281.6 (100.2)	10.3 (5.2)	201.7 (102.3)	4.6 (2.7)	498.3 (210.1)*	0 (0)*	H2	H3
IC1601	62739	1	H2	H2	H2	H2	1.2 (0.6)	136.5 (31.9)	5.6 (1.0)	184.2 (15.8)	4.4 (0.8)	330.8 (42.1)	0 (0)*	H2	H3
Ascocarp 3															
IC1602	62740	2	H2	H2	H2	H2	1.7 (0.5)	353.9 (53.6)	16.8 (4.5)	583.9 (132.2)	16.0 (6.1)	970.6 (189.4)**	0 (0)*	H2	H2
IC1603	62741	1	H1	H1	H1	H2	0.5 (0.1)	209.8 (29.2)	7.5 (1.5)	0 (0)	0 (0)	217.3 (30.6)	130.6 (21.0)**	H1	H5
IC1604	62742	1	H2	H2	H2	H2	2.3 (1.6)	127.9 (38.8)	5.9 (2.0)	260.6 (62.4)	6.4 (2.1)	400.7 (103.6)*	0 (0)*	H2	H3
IC1605	62743	1	H2	H2	H2	H2	1.1 (0.1)	225.4 (10.0)	9.2 (0.9)	362.2 (17.2)	10.0 (1.0)	606.7 (27.3)*	0 (0)*	H2	H3
IC1606	62744	1	H1	H1	H1	H2	1.3 (0.1)	315.8 (6.9)	12.1 (0.5)	0 (0)	0 (0)	328.0 (7.4)	225.5 (8.6)***	H1	H5

Table 3.3. Continued

IC1607	62745	2	H2	H2	H2	H2	2.2 (0.7)	351.0 (49.4)	13.5 (1.7)	390.6 (37.9)	8.3 (0.7)	763.3 (88.8)***	0 (0)*	H2	H2
Parents															
IC278	29507	1	H1	H1	H1	H1	1.5 (0.7)	91.0 (39.5)	2.2 (1.0)	0 (0)	0 (0)	93.2 (40.5)	121.4 (19.0)	H1	H1
IC65	29602	2	H2	H2	H2	H2	346.3 (56.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	H2	H2
Progeny															
Ascocarp															
IC1608	62746	1	H1	H1	H1	H1	0.8 (0.3)	78.2 (20.9)	1.6 (0.4)	0 (0)	0 (0)	79.8 (21.2)**	80.6 (7.3)***	H1	H1
IC1610	62747	2	H1	H1	H1	H1	314.2 (37.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	0 (0)*	H1	H7
IC1611	62748	1	H2	H2	H2	H2	3.5 (0.8)	106.9 (14.9)	5.0 (0.7)	164.2 (12.6)	4.9 (0.3)	280.9 (28.5)***	0 (0)*	H2	H4
IC1612	62749	1	H2	H1	H1	H2	403.1 (18.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	0 (0)*	H3	H3
IC1613	62750	1	H2	H2	H2	H1	0.1 (0.1)	2.5 (1.2)	0 (0)	0 (0)	0 (0)	2.5 (1.2)*	20.4 (3.5)*	H2	H5
IC1614	62751	1	H1	H1	H1	H1	283.6 (18.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	0 (0)*	H1	H1
IC1615	62752	1	H2	H2	H2	H2	108.7 (65.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	138.1 (28.3)**	H2	H4
IC1616	62753	1	H1	H2	H2	H2	396.2 (6.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	0 (0)*	H4	H6
IC1617	62754	2	H2	H2	H2	H2	0.4 (0.3)	202.6 (59.2)	6.7 (2.0)	0 (0)	0 (0)	209.3 (61.0)***	91.4 (16.6)**	H2	H2
IC1618	62755	2	H1	H1	H1	H2	110.6 (73.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	30.4 (26.6)*	H1	H8
IC1619	62756	1	H2	H2	H2	H1	1.7 (0.5)	168.3 (50.2)	5.1 (1.7)	0 (0)	0 (0)	173.3 (51.9)***	116.8 (5.5)**	H2	H5
Parents															
IC278	29507	1	H1	H1	H1	H1	3.0 (1.3)	62.9 (39.3)	1.5 (1.2)	0 (0)	0 (0)	64.4 (40.5)	140.2 (7.4)	H1	H1
IC324	35779	2	H2	H2	H2	H2	310.2 (26.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	H2	H2
Progeny															

Table 3.3. Continued

Ascocarp 1															
IC1620	62757	1	H1	H1	H1	H2	2.2 (1.7)	43.9 (20.0)	0.7 (0.2)	0 (0)	0 (0)	44.7 (20.2)	74.0 (40.0)	H1	H8
Ascocarp 2															
IC1621	62758	1	H1	H1	H1	H1	1.5 (0.4)	190.0 (6.2)	7.6 (0.9)	0 (0)	0 (0)	197.6 (6.7)**	88.2 (17.4)	H1	H1
IC1622	62759	1	H2	H1	H1	H2	10.8 (1.6)	133.6 (38.9)	9.2 (2.9)	433.9 (110.5)	20.6 (5.5)	597.2 (157.8)***	0 (0)*	H3	H3
Ascocarp 3															
IC1623	62760	2	H1	H1	H1	H2	1.6 (0.4)	418.2 (26.2)	18.8 (1.3)	0 (0)	0 (0)	437.0 (27.6)***	249.0 (12.6)***	H1	H7
IC1624	62761	1	H2	H2	H2	H1	218.7 (31.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H4
IC1625	62762	2	H2	H2	H2	H1	122.4 (9.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H5
Ascocarp 4															
IC1626	62763	1	H1	H2	H2	H2	0 (0)	4.5 (2.8)	0.1 (0.1)	0 (0)	0 (0)	4.5 (2.9)	75.4 (11.1)	H4	H6
IC1627	62764	1	H1	H1	H1	H1	4.1 (0.8)	168.0 (78.4)	6.1 (3.0)	0 (0)	0 (0)	174.1 (81.4)**	184.2 (1.4)**	H1	H1
IC1628	62765	1	H1	H1	H1	H2	3.3 (0.7)	257.1 (14.6)	9.5 (0.7)	0 (0)	0 (0)	266.5 (15.3)***	166.7 (27.9)**	H1	H8
Ascocarp 5															
IC1629	62766	1	H1	H1	H1	H1	0.5 (0.1)	173.1 (18.4)	8.3 (1.2)	0 (0)	0 (0)	181.4 (19.5)**	128.5 (10.0)**	H1	H1
IC1630	62767	1	H1	H2	H2	H2	2.3 (0.9)	76.8 (38.5)	2.3 (1.8)	32.5 (29.8)	0.8 (0.9)	112.5 (70.2)	212.5 (102.1)**	H4	H6
IC1631	62768	2	H2	H2	H2	H1	389.6 (28.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)*	H2	H5
Parents															
IC278	29507	1	H1	H1	H1	H1	3.1 (1.2)	114.8 (34.2)	3.7 (1.9)	0 (0)	0 (0)	118.4 (36.1)	183.0 (26.1)	H1	H1
IC33	29570	2	H2	H2	H2	H2	1.3 (0.3)	47.0 (10.2)	1.5 (0.1)	46.4 (5.3)	0.9 (0.2)	95.7 (12.6)	0 (0)	H2	H2

Table 3.3. Continued

Progeny															
Ascocarp 1															
IC1632	62769	2	H2	H2	H2	H2	1.6 (1.2)	52.6 (5.8)	1.2 (0.2)	52.3 (4.4)	0.7 (0.1)	106.7 (9.4)	0 (0)*	H2	H2
IC1633	62770	1	H2	H2	H2	H2	1.6 (0.9)	217.7 (95.0)	9.0 (6.1)	257.8 (161.7)	6.7 (5.1)	491.1 (264.7)	0 (0)*	H2	H3
IC1634	62771	2	H1	H1	H1	H1	2.8 (0.4)	558.7 (66.5)	22.7 (2.9)	0 (0)	0 (0)	581.4 (69.3)***	183.5 (22.4)**	H1	H5
IC1635	62772	1	H1	H1	H1	H2	2.3 (1.5)	63.2 (38.6)	1.7 (1.2)	0 (0)	0 (0)	64.9 (39.8)	66.2 (31.8)***	H1	H6
IC1636	62773	1	H1	H1	H1	H2	3.0 (0.9)	67.6 (30.0)	1.6 (0.9)	0 (0)	0 (0)	69.1 (30.9)	97.5 (25.2)***	H1	H6
Ascocarp 2															
IC1637	62774	1	H1	H1	H1	H2	0 (0)	12.1 (7.3)	0.2 (0.1)	0 (0)	0 (0)	12.3 (7.4)	34.2 (8.4)*	H1	H6
IC1638	62775	1	H2	H2	H2	H2	5.7 (7.6)	221.8 (155.3)	5.0 (5.4)	206.0 (299.2)	3.5 (5.1)	436.4 (464.3)	0 (0)*	H2	H3
IC1639	62776	1	H2	H2	H2	H1	9.7 (1.6)	403.9 (41.5)	11.0 (0.5)	210.6 (5.9)	3.9 (0.3)	629.4 (46.6)***	0 (0)*	H2	H4
IC1640	62777	1	H2	H2	H2	H2	1.5 (0.4)	83.1 (9.6)	2.5 (0.8)	107.5 (42.1)	2.1 (1.1)	195.1 (52.1)	0 (0)*	H2	H3
IC1641	62778	1	H1	H1	H1	H2	3.9 (1.7)	74.0 (24.9)	1.4 (0.3)	0 (0)	0 (0)	75.4 (25.2)	122.5 (9.4)***	H1	H6
IC1642	62779	1	H2	H2	H2	H2	0.8 (0.2)	82.8 (14.9)	1.5 (0.1)	48.9 (10.3)	0.7 (0.1)	133.9 (11.4)	0 (0)*	H2	H3
IC1643	62780	1	H2	H2	H2	H1	2.6 (1.8)	257.0 (34.9)	10.2 (0.9)	516.9 (72.9)	12.4 (1.4)	796.5 (109.9)***	0 (0)*	H2	H4

^aParental mating-type (*MAT*) designations for NRRL29602, 35779 and 35782 are from Ramirez-Prado *et al.* (2008); parental mating-type (*MAT*) designations for NRRL29507 and 29570 are from Horn *et al.* (2009a) and Horn *et al.* (2009b), respectively.

^bStrain numbers (NRRL) from Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

^cIndicates mean aflatoxin production and (standard deviation).

^dIndicates mean *O*-methylsterigmatocystin production (OMST) and (standard deviation).

^eIndicates mean cyclopiazonic acid (CPA) production and (standard deviation).

^fCluster MLST refers to the collapsing of DNA sequences comprising the loci within the aflatoxin cluster (chr. 3).

Table 3.3. Continued

^aGenome MLST incorporates all loci examined into the collapsing of DNA sequences.

^bFor each cross, ascospores from different ascocarps were examined separately. Individual progeny ascospores are designated as Parent 1 × Parent 2-ascocarp number-ascospore number.

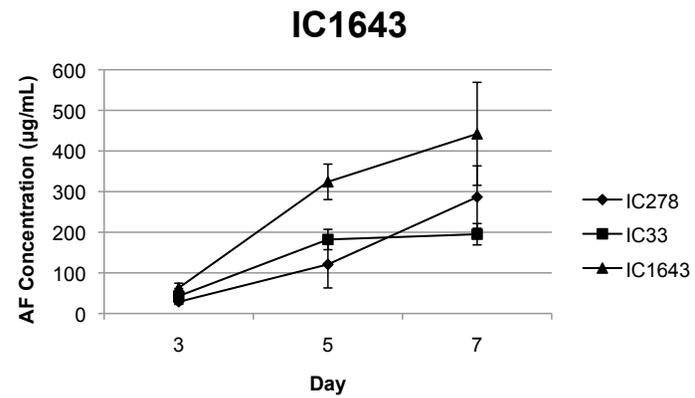
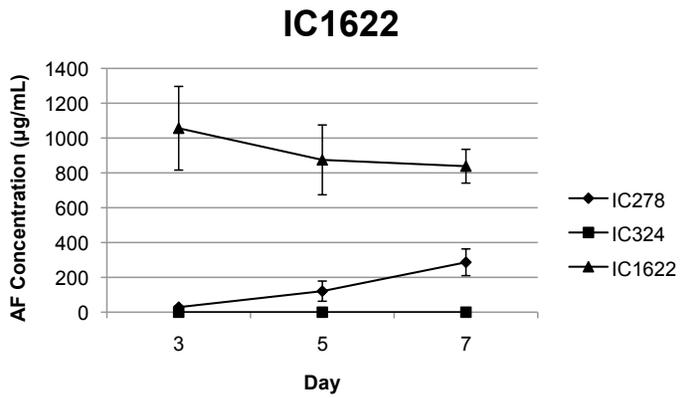
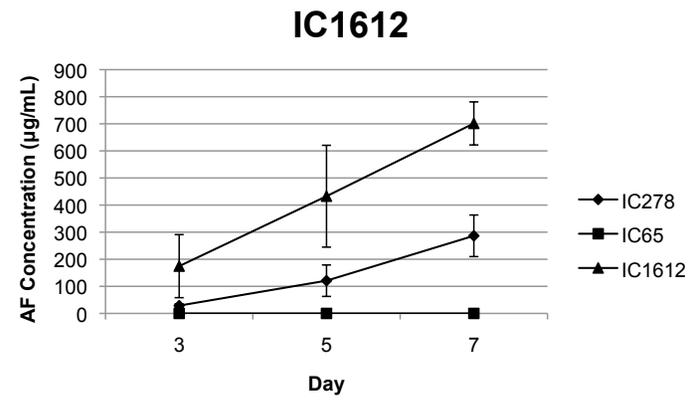
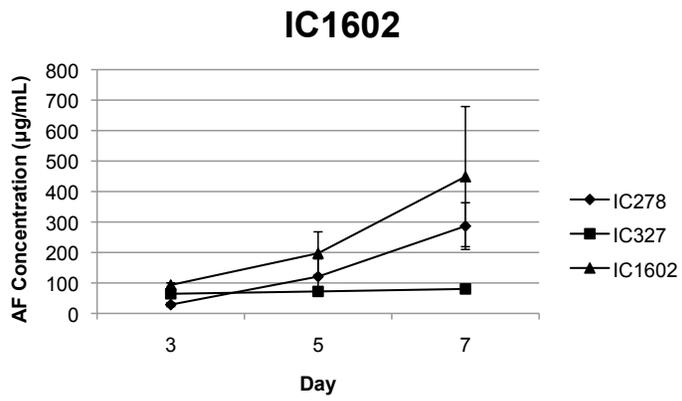
*Indicates that total AF and CPA concentration values are statistically different from the *A. flavus* parent (IC278) for that particular cross.

**Indicates that total AF and CPA concentration values are statistically different from the *A. parasiticus* parent (IC327, IC65, IC324 or IC33) for that particular cross.

***Indicates that total AF and CPA concentration values are statistically different from either parent in that particular cross.

Figure 3.3. Aflatoxin concentration curves showing total aflatoxin production in four progeny strains compared to parental strains after 3, 5 and 7 days of growth.

Aflatoxin Concentration Curves



Ploidy

Although *A. flavus* and *A. parasiticus* conidia have been reported to be multinucleate, FACS analysis showed that conidial populations of parental strains are haploid with an approximately even split of monokaryons and dikaryons. All progeny strains were found to be haploid recombinants rather than heterozygous diploids of the two parental strains (Figure S3.1).

MLSTs

As confirmed by MLST genotyping, 77% (34/44) of the progeny were recombinants via independent assortment, whereas only one progeny strain (IC1606) was a recombinant in the AF gene cluster (Table 3.2). Unlinked loci segregated in a Mendelian fashion and showed independent assortment. For example, in cross IC278 × IC65, the MLSTs (Table 3.2) showed that progeny isolate IC1612 has the genetic background of the *A. flavus* IC278 parent for the *MAT* marker on chromosome 6, and inherited chromosome 4 from the *A. parasiticus* IC65 parent. Moreover, one of the markers (*aflC/aflD*) on chromosome 3 is decoupled from the other two (*aflM/aflN* and *aflW/aflX*) such that a putative crossover event is inferred to have occurred somewhere between the *aflC/aflD* and *aflM/aflN* regions.

Although there was a significant difference in the frequency of *MATI-1* (75%) and *MATI-2* (25%) in the progeny when pooled across all crosses ($n = 44$, $P = 0.0012$), progeny from each cross tested separately showed no significant deviation from 1:1, as would be expected for any two segregating loci that are able to assort independently during meiosis. Similarly, we examined segregation of AF cluster genes *aflC/aflD*, *aflM/aflN*, and *aflW/aflX*,

on chromosome 3 and *trpC* on chromosome 4. With the exception of *trpC* in cross IC278 × IC327 where H2 predominated, there were no significant differences in the frequency of the two parental alleles in the progeny. All sequence data were submitted to GenBank under Accession numbers KF164617 – KF164813.

Genome-wide dendrogram and aflatoxin cluster heat maps

Clustering based on array data revealed three distinct clades: *A. parasiticus*-only clade; a clade that includes *A. minisclerotigenes*, *A. nomius* and experimental *A. flavus* × *A. parasiticus* hybrids; and an *A. flavus* L strain clade that includes *A. oryzae* (Fig. 3.4). The L strain clade was further subdivided into groups IA, IB, and IC according to Geiser *et al.* (2000). One experimental hybrid progeny strain IC1630 showed very close similarity to *A. minisclerotigenes* sampled from Benin (IC1112, IC1142), *A. flavus* S from Georgia (IC1169) and Australia (IC720, IC796) and *A. nomius* from Louisiana (IC1516). The corresponding heat maps for chromosome 3R are shown in Figure 3.5. In *A. flavus* L, the heat maps show patterns of deletions in the cluster consistent with Geiser's group IB (Geiser *et al.* 1998), whereas experimental hybrids show a distinct hybridization pattern with similarity to *A. parasiticus* and *A. flavus* group IC (IC278) in the cluster; one hybrid strain (IC1630) is more similar to group II which includes *A. flavus* S, *A. minisclerotigenes* and *A. nomius*; the hybridization pattern for nonaflatoxigenic *A. caelatus* and *A. tamaritii* is more *A. parasiticus*-like with close affinity to group II (Fig. 3.5).

Figure 3.4. Genome-wide similarity dendrogram based on aCGH data of a representative sample of fifty-six progeny strains representing variation in intra and interspecific crosses, as well as isolates sampled from populations of *A. flavus* and *A. parasiticus* and isolates from closely related species in *Aspergillus* section *Flavi*. Red squares indicate high correlation in microarray hybridization signal intensities, which translates to high DNA sequence similarity among individuals in a particular grouping, while blue indicates the lowest level of similarity. Hybrid progeny individuals and clades are denoted with red lettering and lines, respectively. Individuals are grouped according to similarity with respect to the *A. flavus* NRRL 3357 reference strain.

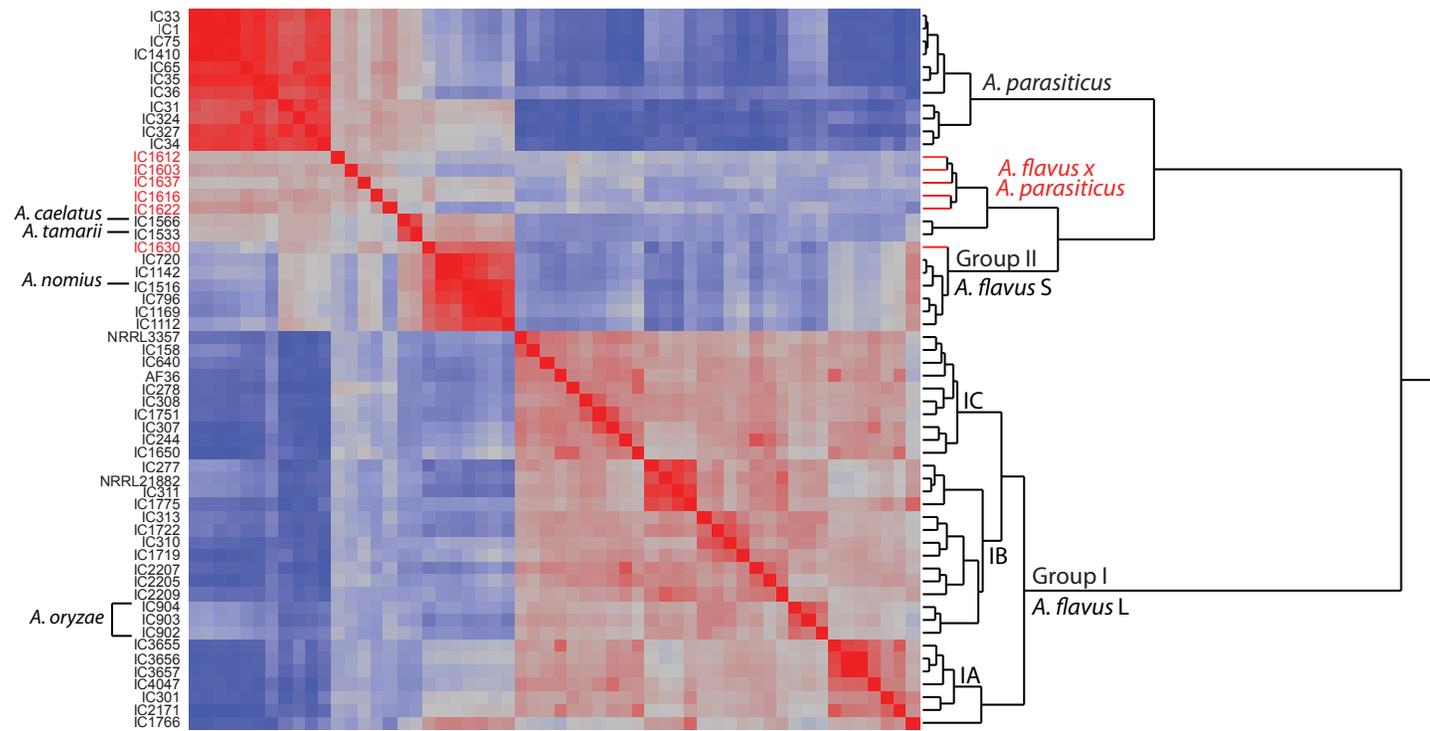
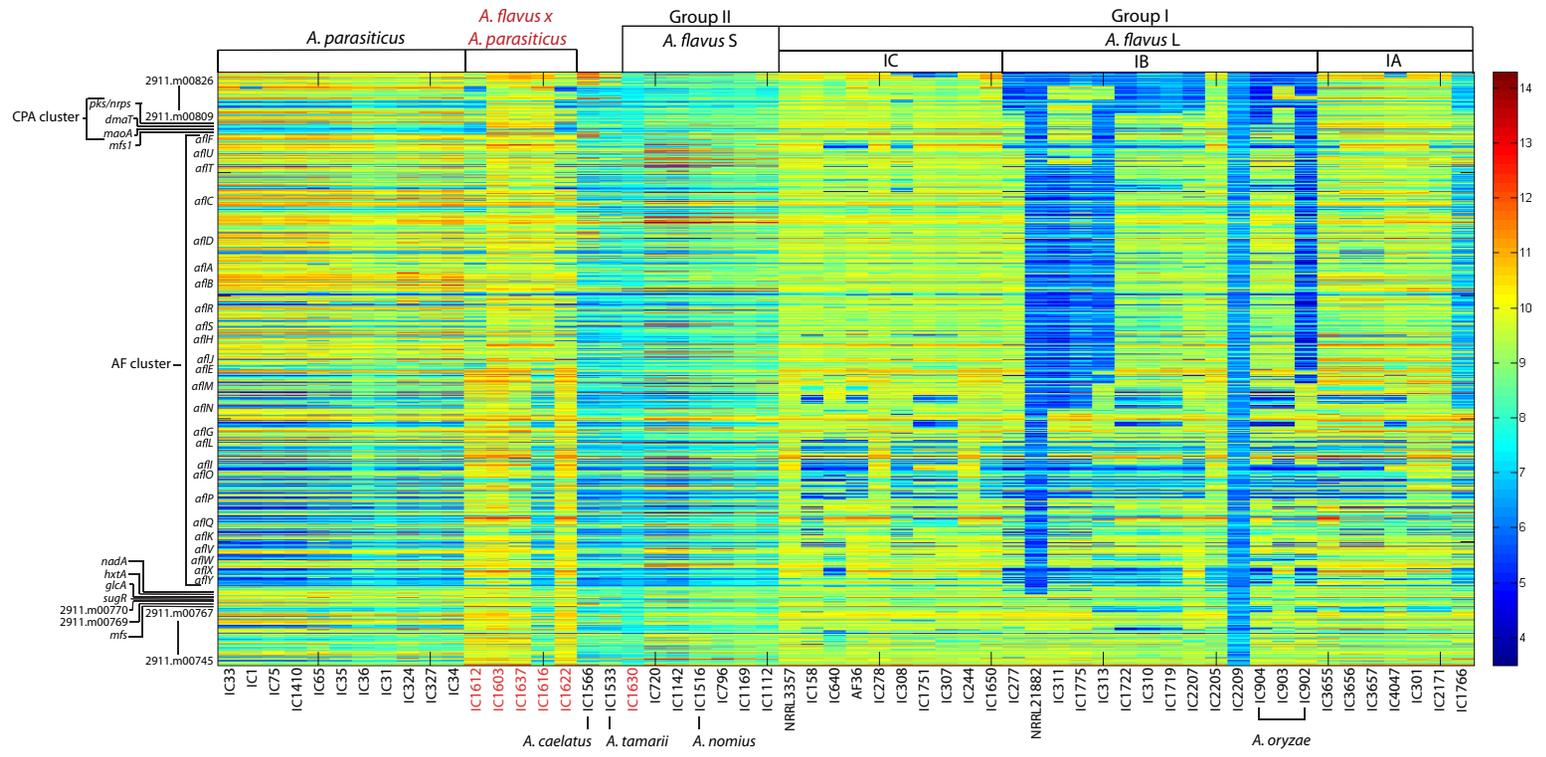


Figure 3.5. Heat maps based on aCGH of only the right arm of chromosome 3 for the 56 strains in Fig. 4; the strains are ordered based on clades inferred from the dendrogram in Fig. 4. The genes in the CPA, aflatoxin and sugar clusters are labeled. Red bars indicate the highest level of hybridization of the isolate to reference strain *A. flavus* NRRL 3357 in a particular gene region. Blue bars indicate the lowest level of hybridization to the reference strain. Yellow, green and aqua indicate intermediate levels of hybridization.



Parent-offspring trio heat maps

Heat maps of six parent-offspring trios identified putative crossovers in the AF cluster of three progeny based on single feature polymorphisms (SFPs) (Fig. 3.6). All three recombinant hybrid strains IC1612, IC1616 and IC1622 arose from a single crossover in the *aflE* and *aflE/aflM* intergenic region in chromosome 3R (Figs. 3.5, 3.6). The cluster-specific genetic markers *aflC/aflD*, *aflM/aflN* and *aflW/aflX* span the recombination breakpoint and confirm the aCGH results (Table 3.2). All aCGH data were submitted to the Gene Expression Omnibus (GEO) under series accession number EED51121.1 - EED51201.1.

Genome-wide parentage plots

Both independent assortment and crossovers were observed in genome-wide aCGH parentage plots for the six parent-offspring trios (Fig. 3.7). Sequence data from linked loci in the AF cluster (*aflC/aflD*, *aflM/aflN* and *aflW/aflX*) and unlinked loci (*MAT*, *trpC*) comprising the MLST confirmed that patterns of inheritance observed in parentage plots are the result of SFPs between the labeled target DNA and *A. flavus* NRRL 3357 probes fixed on the array. Known patterns of inheritance from sequenced MLST loci (position indicated with vertical blue lines in parentage plots) confirm inferred independent assortment and crossover events in the parentage plots. For example, the *trpC* sequence on chromosome 4 in progeny IC1612 is identical to the *A. parasiticus* parent IC65, whereas *MAT1-1* on chromosome 6 is from the *A. flavus* parent IC278 (Table 3.2, Fig. 3.7B).

In the absence of crossovers, chromosomal inheritance in progeny isolates can be deduced by examining parentage plots. An example illustrating this is chromosome 7 in

Figure 3.6. Heat maps of six *A. flavus* and *A. parasiticus* parent-offspring trios for the same chromosomal region shown in Figure 5. In each trio plot, the center plot is the progeny individual, flanked on the left side by the *A. flavus* parent and on the right side by the *A. parasiticus* parent. Red bars indicate the highest level of hybridization of the isolate to reference strain *A. flavus* NRRL 3357 in a particular gene region. Blue bars indicate the lowest level of hybridization to the reference strain. Yellow, green and aqua indicate moderate levels of hybridization. The blue line between plots is an indication of greater similarity to either the *A. flavus* parent (approaching 1.0) or greater similarity to the *A. parasiticus* parent (approaching 0.0.) Black horizontal bars indicate putative recombination breakpoints where progeny hybridization patterns transition from one parent to the other.

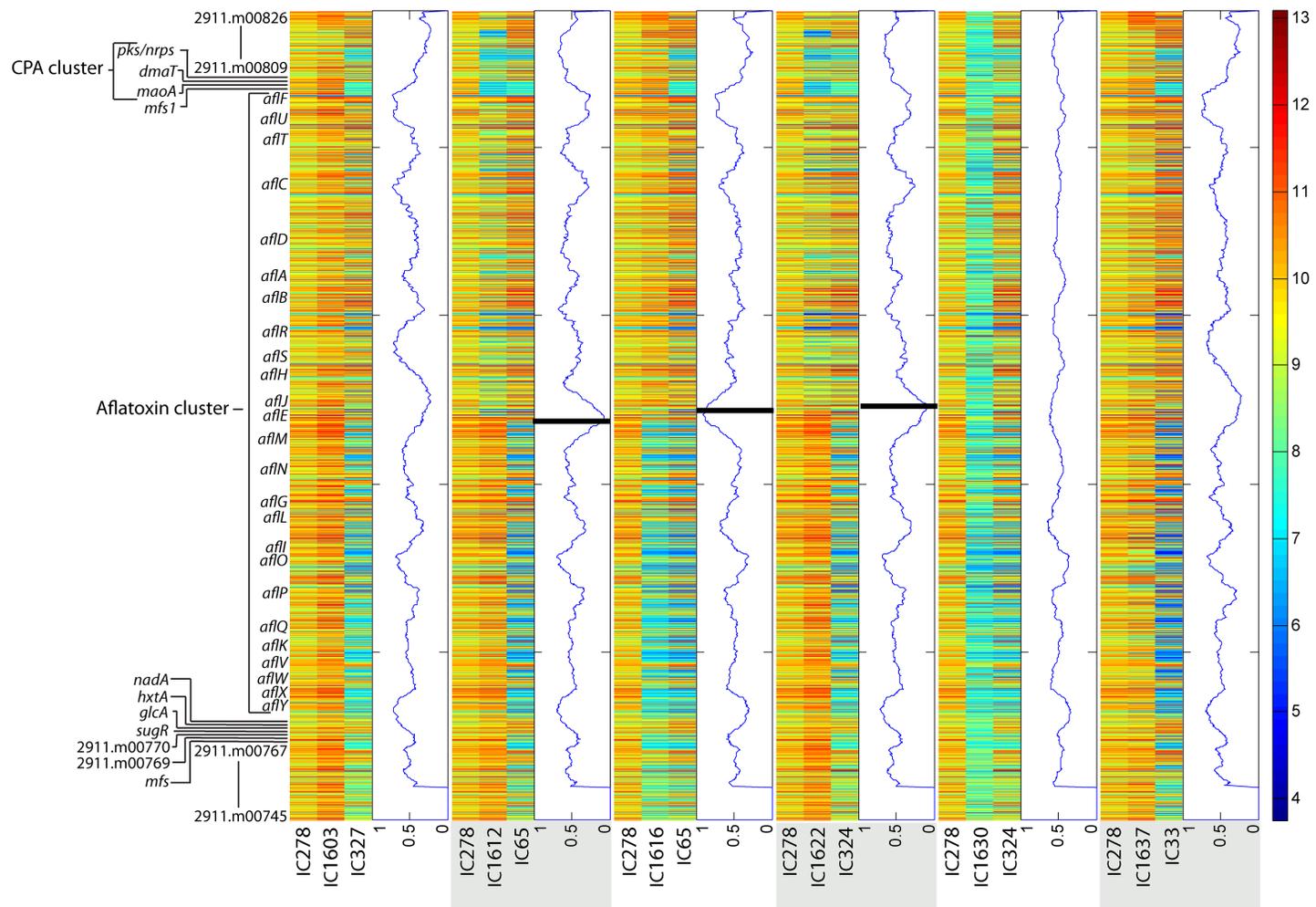
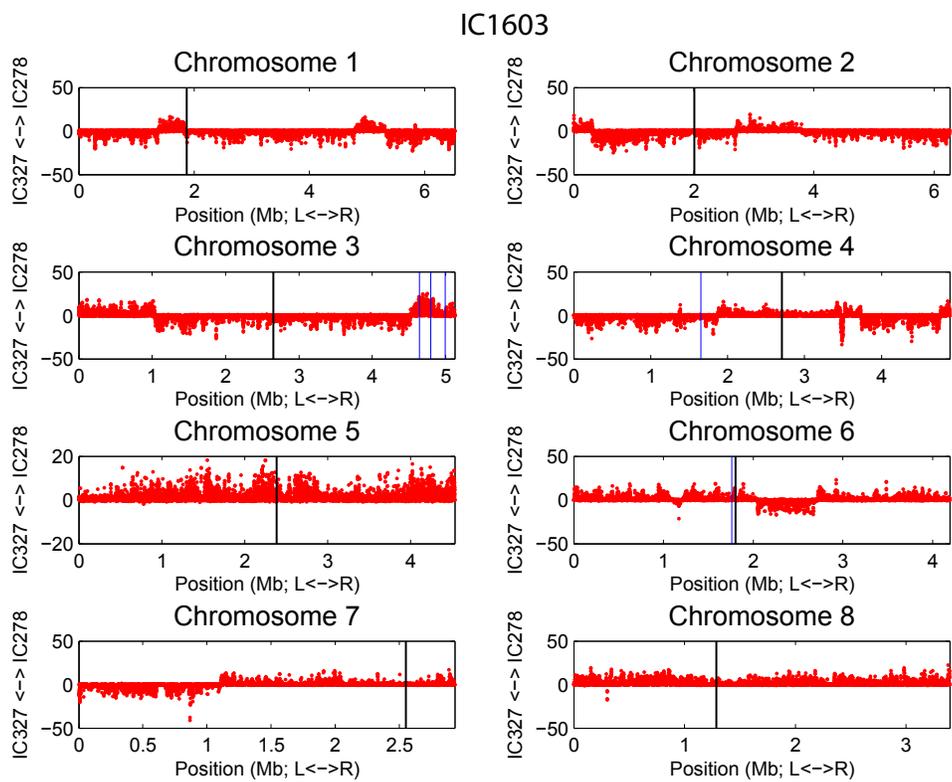


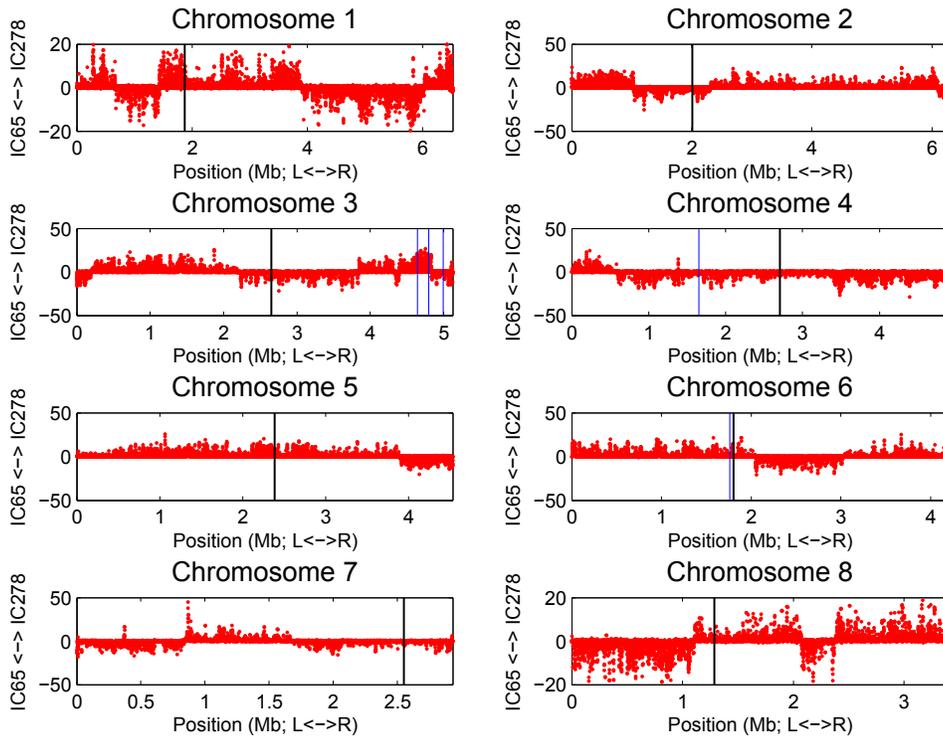
Figure 3.7. Genome-wide parentage plots for the six hybrid progeny shown in Figure 6. The progeny strains are labeled from A-F. Each red point represents a probe on the array. Dots with positive values on the y-axis indicate a hybridization pattern more similar to the *A. flavus* parent; dots with negative values indicate a hybridization pattern more similar to the *A. parasiticus* parent. Black vertical lines represent the putative centromere location on each chromosome. Blue lines represent MLST loci: *aflC/aflD*, *aflM/aflN* and *aflW/aflX* on chromosome 3, *trpC* on chromosome 4 and *MAT* on chromosome 6.

A



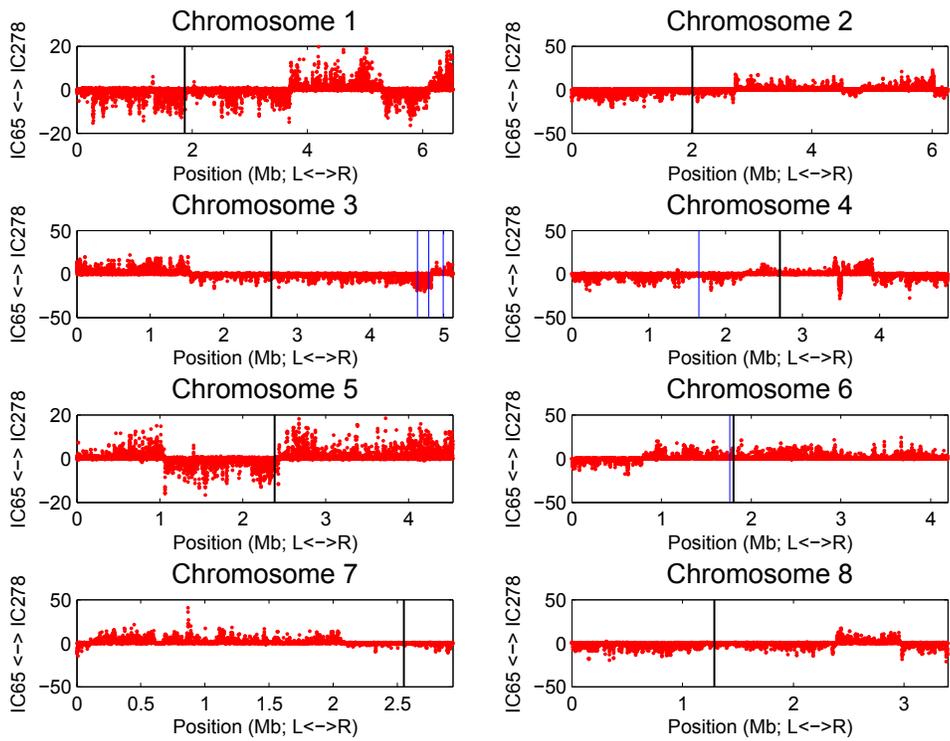
B

IC1612



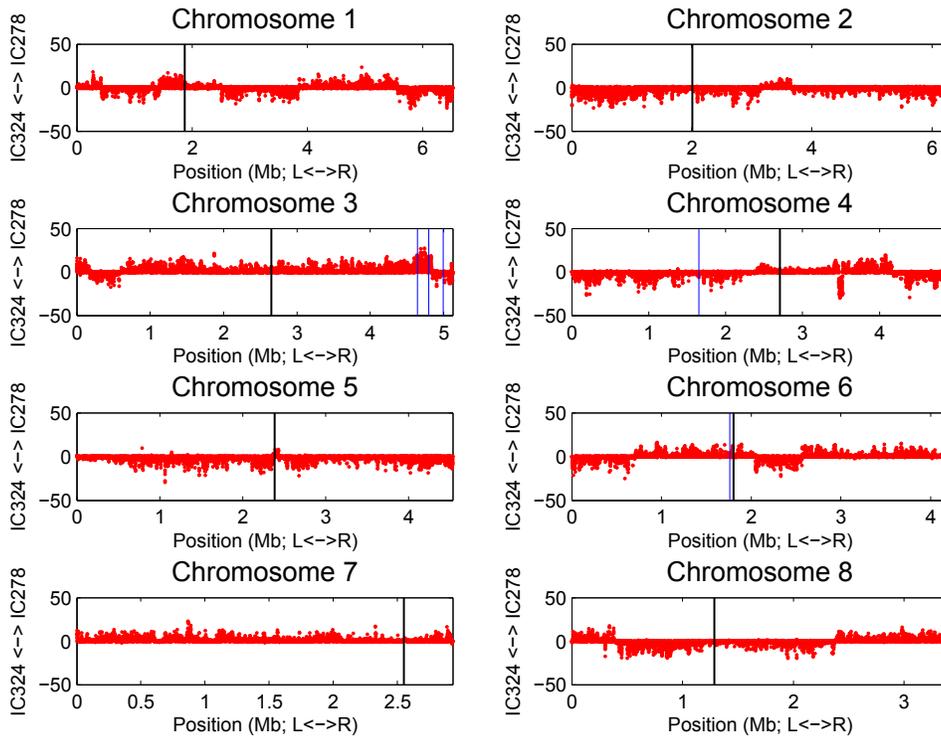
C

IC1616



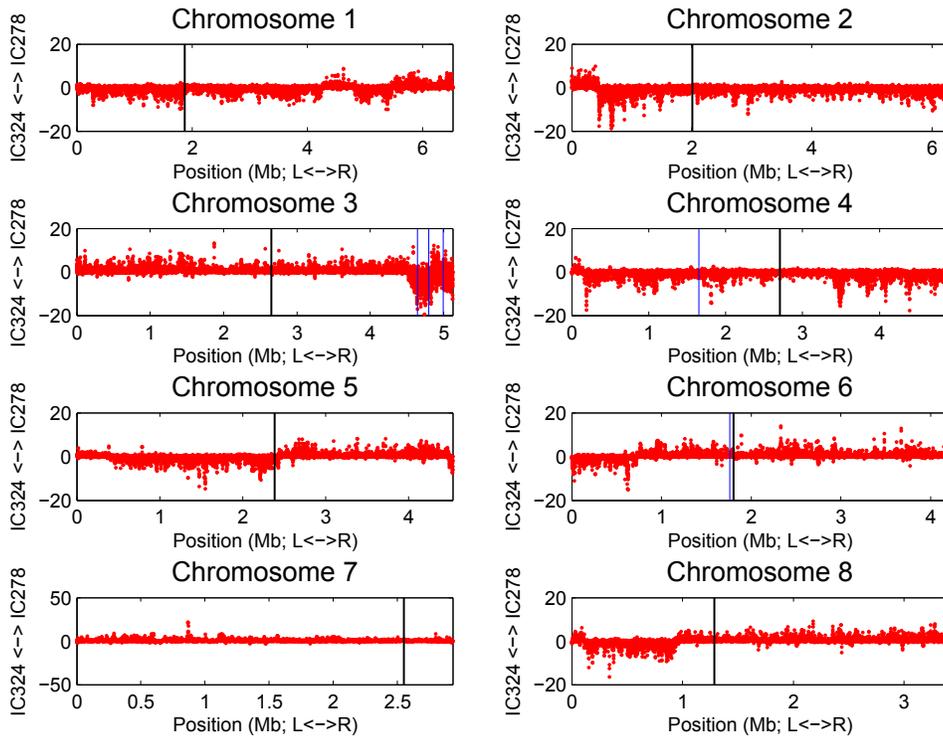
D

IC1622



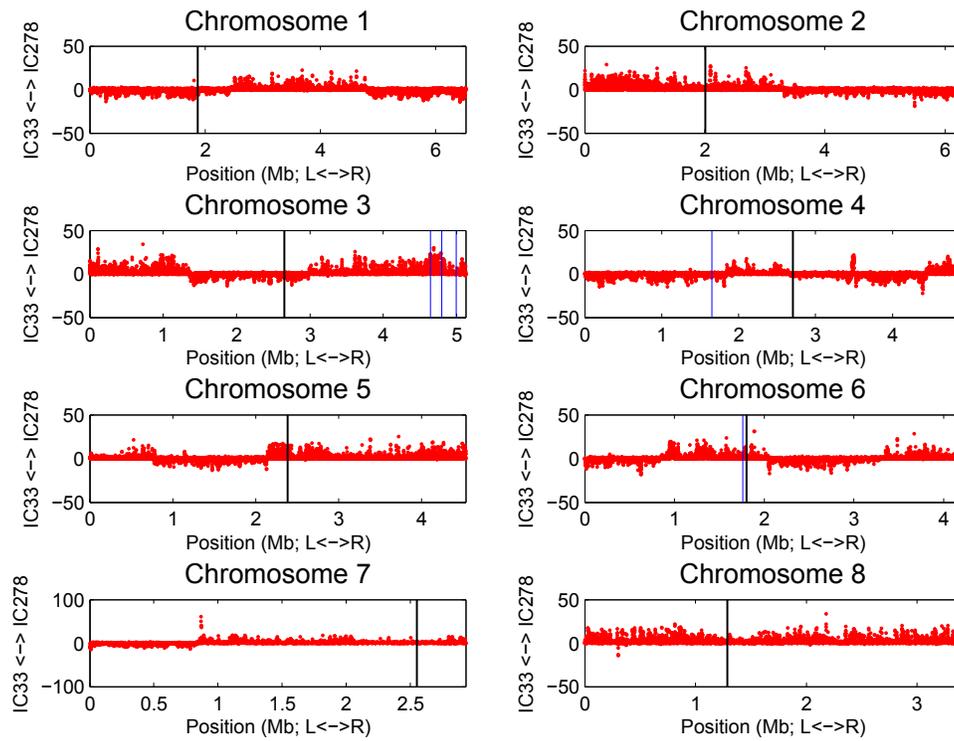
E

IC1630



F

IC1637



progeny strain IC1622 from cross IC278 × IC324 (Fig. 3.7D). There is evidence of stronger hybridization above the x-axis than below, which indicates that chromosome 7 in IC1622 has the genetic background of the *A. flavus* IC278 parent. The parentage plots also reveal frequent crossover events resulting in progeny having chromosomes of hybrid origin. Crossovers were predominantly located in subtelomeric regions. For example, in the AF gene cluster for IC1622, the *aflC/aflD* region of chromosome 3R is from *A. parasiticus* parent IC324, while the *aflM/aflN* and *aflW/aflX* regions (leftmost two vertical blue lines) are from *A. flavus* parent IC278, suggesting one crossover in chromosome 3R; the subtelomeric end of the left arm of chromosome 3 also shows evidence of a crossover but in this case IC1622 is more similar to *A. flavus* IC278 (Fig. 3.7D). In some interspecific progeny, crossovers were localized to centromeric regions of chromosomes. For example, IC1630 inherited the right arm of chromosome 5 from the *A. flavus* IC278 parent and the left arm from the *A. parasiticus* IC324 parent (Fig. 3.7E). In IC1616 and IC1603, crossovers also coincide with the putative location of centromeres on chromosomes 5 (Fig. 3.7C) and 1 (Fig. 3.7A), respectively.

Based on the spacing of probes between adjacent genes on the *A. flavus* microarray, the shortest interval for detecting recombination breakpoints was ~15 kb. The higher probe density in the tiled intergenic regions of the AF cluster allowed for more accurate detection of recombination breakpoints; the dissimilarity between *A. parasiticus* and the *A. flavus* oligonucleotide-based probes on the arrays further permitted visual detection of recombination breakpoints genome-wide. Overall at least one crossover event per

chromosome arm was observed, with double and triple crossovers more common on longer chromosome arms such as, for example, on chromosome 1R (Fig. 3.7).

Principal component analysis and phylogenetic inference

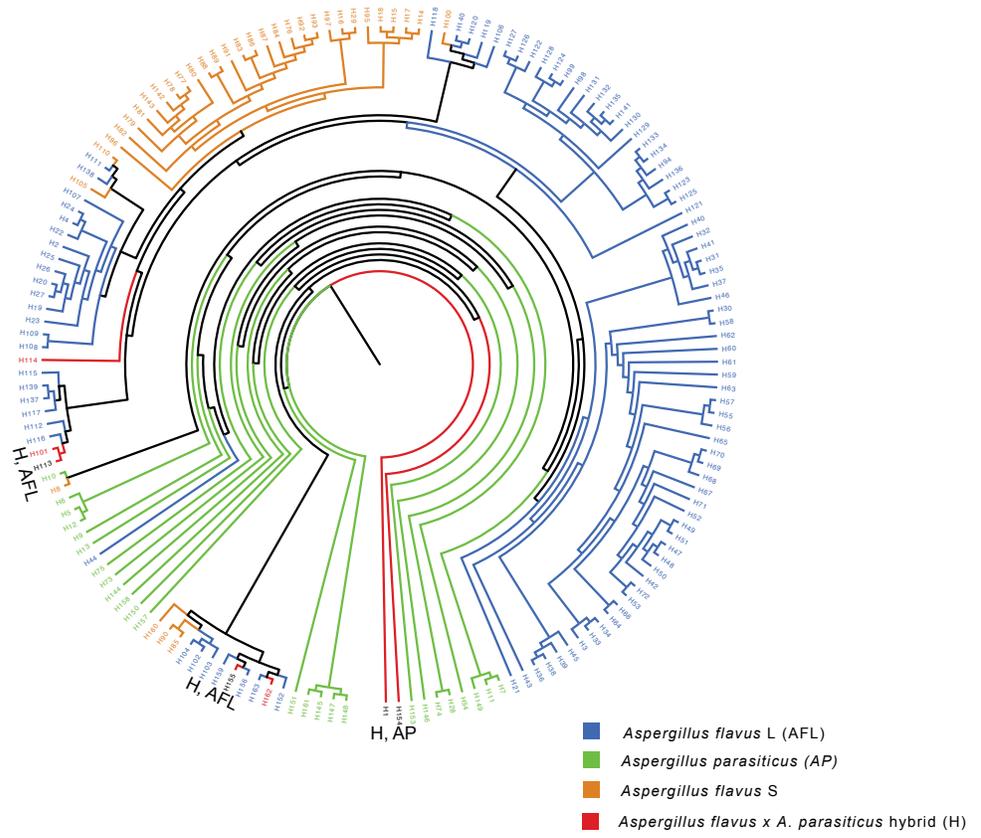
Maximum likelihood bootstrap analysis (Fig. 3.8A) revealed that the hybrid strains share haplotypes with *A. parasiticus* (haplotypes H1 and H154) and *A. flavus* L (haplotypes H101 and H113); moreover, hybrid haplotypes H155 and H162 also share a recent common ancestor with *A. minisclerotigenes* (haplotypes H85, H90 and H160) (Table S3.2). PCA and the Tracy-Widom statistic identified a total of three significant axes of variation in the global MLST data (Fig. 3.8B). Further applying Tibshirani's gap statistic revealed at least three significant clusters: a group including all *A. parasiticus* isolates, an *A. flavus* L group, and a third distinct group including *A. flavus* S_B and *A. minisclerotigenes* from Australia and Benin. Experimental hybrid strains were tightly clustered with natural *A. flavus* and *A. parasiticus* isolates from populations in the United States, Africa, Argentina, Australia and India.

Discussion

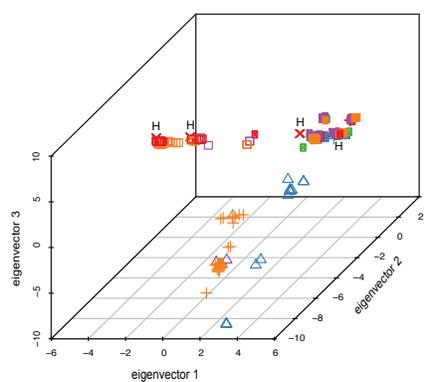
We present direct genetic evidence of interspecific hybridization between *A. flavus* and *A. parasiticus*. We also show that interspecific hybrids show enhanced aflatoxin production. Several mechanisms may be involved that increase aflatoxin concentrations in hybrid progeny. One possibility involves variation in patterns of DNA methylation in the aflatoxin gene cluster and genome-wide. DNA methylation contributes to epigenetic processes, which play a fundamental role in organismal adaptation to different environmental conditions

Figure 3.8. Maximum likelihood phylogenetic (A) and principle component (B) analyses based on three MLST loci (*aflM/aflN*, *aflW/aflX* and *trpC*) for experimental hybrid strains and natural isolates of *A. parasiticus*, *A. flavus* L, S_B, and *A. minislerotigenes* (S_{BG}) sampled from populations on a global scale. Symbols in the PCA plots denote the species and colors indicate geographical location.

A Global Phylogeny



B Principal Component Analysis



- | | |
|--|------|
| □ <i>A. parasiticus</i> | ■ US |
| ⊠ <i>A. flavus</i> L | ■ BE |
| △ <i>A. minisclerotigenes</i> | ■ IN |
| + <i>A. flavus</i> S _B | ■ AR |
| × <i>A. p.</i> x <i>A. f.</i> hybrid (H) | ■ AU |

(Angers *et al.* 2010). Because DNA methylation signatures are transmitted across generations and are highly heritable, modifications of these patterns as a result of crossovers and independent assortment may relieve or activate repressors or other regulators of aflatoxin production. For example, hybrid offspring IC1603 and IC1637 have significantly higher and lower mean aflatoxin B₁ concentrations, respectively, than the parental *A. flavus* IC278 strain. In both IC1603 and IC1637, there is no evidence of crossovers in the aflatoxin cluster, which suggests that the factors modulating toxicity are located elsewhere in the genome. The possibility of using interspecific crosses for genetic mapping of loci that down- and un-regulate toxicity warrants further investigation.

Hybridization has been suggested to be a means of creating diversity in a population, or an entirely new species altogether (Mallet 2007). Although successful hybrids have been reported in fungi (Stukenbrock *et al.* 2012), hybridization is usually selected against because hybrid fitness is lower than that of pure populations (Dettman *et al.* 2007). Morphologically, progeny from interspecific *A. flavus* × *A. parasiticus* differed from their parents in growth rate, sclerotia production, stipe length, conidial head seriation and conidial features (size, ornamentation, color) (Table 3.1). The degree of fitness imparted by these phenotypic characters is not known. Hybridization also showed a significant qualitative and quantitative influence on toxicity. Moreover, the high heritability of both AF and CPA in interspecific crosses indicates that genetic variation in the parents, in the form of mutations and recombination, has a direct effect on aflatoxin diversity and aflatoxin quantities, which could be important fitness components. For example, the casual species responsible for an outbreak of human aflatoxicosis in Kenya in 2004 was found to produce only B or B + G

aflatoxins and to be more similar to the B and G producing *A. minisclerotigenes* than to the *A. flavus* S_B morphotype (Probst *et al.* 2012; Probst *et al.* 2007; Probst *et al.* 2010). This is not surprising in light of the potential for hybridization between *A. flavus* and *A. parasiticus* and the XXX. Interestingly, the outbreak strain was not isolated from maize or soil in the affected Kenyan districts prior to 2004 and all isolates from the outbreak produced much higher quantities of aflatoxin B₁ than sympatric L strain isolates (Probst *et al.* 2010). Whether this is an adaptive hybrid strain showing heterosis in toxin production or an introduced strain is unknown, but it is clear that this outbreak strain is better able to colonize soil and infect maize compared to sympatric *A. flavus* L and *A. parasiticus*. Moreover, the incidence of *A. parasiticus* is very low in the affected Kenyan districts, which may reflect a decrease in its fitness relative to the putative hybrid strain that has characteristics of both parental species, and is therefore better able to thrive in soil and maize environments.

Our results indicate that recombination as a result of hybridization may be important in the genesis of novel hybrid toxin profiles. For example, two progeny (IC1611 and IC1622) synthesized G₁ and G₂ aflatoxins that were not produced by either parent. In both cases, G aflatoxin function was rescued as the result of a crossover event between *A. flavus* (IC278) and *A. parasiticus* (IC65 or IC324) midway in the cluster close to *aflE* (Fig. 3.6), such that the region from *aflE* to the telomere was inherited from the OMST producing *A. parasiticus* parent. This indicates that the loss of function mutation in these OMST producing *A. parasticus* strains is somewhere in the *aflE-nadA* late pathway segment of the cluster. A previous study reported that *orda* (=aflQ), *cypA*(=aflU) and *nadA* are important in the conversion of OMST to G aflatoxins (Zeng *et al.* 2011). Since *aflU* is in the *A.*

parasiticus subtelomeric segment, this suggests that *aflU* is functional in IC1612 and IC1622 and that a possible loss of function mutation resides in *aflO*, *nadA*, or both. Crossover events involving the inheritance of the telomeric end from the *A. flavus* parent can also result in hybrid progeny that produce CPA in addition to B and G aflatoxins, a mycotoxin profile not typical of *A. flavus* or *A. parasiticus*. This is illustrated by IC1630, which originated from the same cross as IC1622 (Table 3.3), but we would expect to find more progeny with a similar toxin profile with a larger sampling of offspring.

According to the genotyping results (Table 3.2), progeny strain IC1630 shares the same haplotype as parental strain IC324 in the *aflM/aflN* and *aflW/aflX* regions, which then transitions to a haplotype shared by parental strain IC278 in the *aflC/aflD* region such that IC1630 synthesizes B and G aflatoxins, as well as CPA. Moreover, the trio heat map of IC278, IC1630 and IC324 (Fig. 3.6) revealed a unique hybridization phenotype for IC1630 similar to that observed for strains of *A. minisclerotigenes*, *A. nomius*, *A. tamarii*, and *A. caelatus* (Fig. 3.5). A closer inspection of the corresponding aCGH parentage plot of this trio shows high hybridization signal intensity in the subtelomeric segment of chromosome 3R (Figure 3.7E) that is in the direction of both parents, particularly in the telomeric end. This signature was not observed elsewhere in the genome of IC1630 and there was no evidence of this phenotype in the heat maps of chromosome 3R of other hybrid progeny (Figure 3.5). A similar phenomenon was observed in *A. flavus* intraspecific crosses (Olarie *et al.* 2012) where it was attributed to the existence of genetically heterogeneous nuclei that are heterokaryotic for the aflatoxin gene cluster. We hypothesize that the nuclei are genetically unbalanced with respect to the right arm of chromosome 3, and as a result the cluster copy

number is lower than adjacent regions. Clearly the genetic and toxin phenotype data indicate that a crossover occurred between the aflatoxin clusters of *A. flavus* and *A. parasiticus* but the resultant recombinant may be present in less than single copy levels making it difficult to resolve using aCGH. Examining additional genetic markers in the aflatoxin and CPA clusters of IC1630 may further elucidate the recombination structure of this hybrid offspring. Whether copy variation in the cluster plays a role in regulating aflatoxin biosynthesis and facilitating adaptation in these fungi warrants further investigation. These data indicate that there may be several different mechanisms that generate toxin diversity in these fungi.

The potential of hybridization in these fungi may also impact their fertility. As reported previously in *A. parasiticus* (Horn *et al.* 2009b) and *A. flavus* (Horn *et al.* 2009a), the same parental strain in different crosses yielded different fertilities. For example, *A. parasiticus* IC65 had the highest fertility when crossed with *A. flavus* IC278 and the lowest fertility when crossed with IC314 (Table 3.1). In this study, all four interspecific crosses that produced viable progeny involved *A. flavus* IC278 and it is therefore possible that the *A. flavus* parental strain is driving fertility in interspecific crosses. Previous work showed that IC65 is possibly heterokaryotic for mating type (Carbone, unpublished data). Whether heterokaryosis arising from hybridization plays a role in fertility is unknown and requires additional study.

Results from PCA and phylogenetic analysis suggest that hybrid offspring are polyphyletic, showing tight clustering and common ancestry with *A. flavus* L- and S- strains and *A. parasiticus*. The clustering of S_B and *A. minisclerotigenes* suggests either recent population admixture or shared common ancestry. Hybrid progeny strains appear to cluster

with chemotype specific toxin classes within each species and morphotype. For example, hybrid progeny IC1630, which produces B and G aflatoxins and CPA, groups with IC1112, an *A. minisclerotigenes* strain from Benin with a similar toxin profile. In the maximum likelihood DNA-sequence based phylogeny (Fig. 3.8A), IC1630 shares a haplotype (H154) with other naturally occurring *A. parasiticus* OMST producers (Table S3.2) even though genome-wide it has very close affinities to *A. minisclerotigenes* (Fig. 3.4). In a previous study we showed that the *hypE* gene, which is located in the *aflM/aflN* intergenic region, might be a target of balancing selection acting to maintain G₁-dominant and OMST specific classes in *A. parasiticus* (Carbone *et al.* 2007). The recent shared common ancestry of *A. parasiticus* G₁ dominant strains and *A. flavus* L based on variation in *hypE* supports the possibility of interspecific hybridization giving rise to G₁ dominant strains in *A. parasiticus*. PCA analysis in Figure 3.8B shows that hybrids are positioned at extremes within each *A. parasiticus* and *A. flavus* species cluster, which indicates that they may be responsible for the genetic and toxin heterogeneity observed in natural populations. For example, high aflatoxin-producing (600-1000µg/mL total aflatoxin) hybrid progeny IC1602 and IC1643 (Table 3.2; Fig. 3.3) are tightly clustered with other high aflatoxin producing *A. parasiticus* and *A. flavus* strains sampled from nature. Further examination of interspecific hybrids may yield additional insights into the evolution of toxicity in these agriculturally important species.

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Data Accessibility

DNA sequences: Genbank accessions KF164617 – KF164813

aCGH data: Gene Expression Omnibus (GEO) accessions EED51121.1 - EED51201.1

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APPENDICES

Appendix A

Table S3.1. Species and source of strains for XXX

Species	IC strain	NRRL strain	Source
<i>Aspergillus parasiticus</i>	IC1	29538	Georgia
	IC33	29570	Georgia
	IC31	29568	Georgia
	IC34	29571	Georgia
	IC35	29572	Georgia
	IC36	29573	Georgia
	IC65	29602	Georgia
	IC75	29612	Georgia
	IC324	35779	Georgia
	IC327	35782	Georgia
	IC1410	62733	Experimental
<i>Aspergillus flavus</i>	IC158	1957	South Pacific
	IC201	21882	Georgia
	IC244	29473	Georgia
	IC277	29506	Georgia
	IC278	29507	Georgia
	IC301	29530	Georgia
	IC307	29536	Georgia
	IC308	29537	Georgia
	IC310	35736	North Carolina
	IC311	35737	Alabama
	IC313	35739	Texas
	IC640	62727	Australia
	IC720	62728	Australia
	IC796	62729	Australia
	IC899	3357	USA
	IC1112	62730	Benin
	IC1142	62731	Benin
	IC1169	62732	Georgia
	IC1179	18543	Arizona
	IC1650	62315	Experimental
	IC1719	62347	Experimental
IC1722	62350	Experimental	
IC1751	62390	Experimental	
IC1766	62358	Experimental	
IC1775	62367	Experimental	

Table S3.1. Continued

	IC2171	62374	Experimental
	IC2205	62407	Experimental
	IC2207	62409	Experimental
	IC2209	62411	Experimental
	IC3655	30797	Mississippi
	IC3656	58987	Mississippi
	IC3657	30796	Mississippi
	IC4047	35743	Georgia
<i>A. flavus</i> x <i>A. parasiticus</i>	IC1603	NULL	Experimental
	IC1612	NULL	Experimental
	IC1616	NULL	Experimental
	IC1622	NULL	Experimental
	IC1630	NULL	Experimental
	IC1637	NULL	Experimental
<i>A. caelatus</i>	IC1566	26111	Georgia
<i>A. tamarii</i>	IC1533	26078	Georgia
<i>A. nomius</i>	IC1516	26886	Louisiana
<i>A. oryzae</i>	IC902	ATCC 22786	Japan
	IC903	ATCC 27788	?
	IC904	5590	Japan

Appendix B

Table S3.2. Haplotypes and strain designations for haplotypes shown in Figure 3.8A

Haplotype	Strain
H1	IC100, IC101, IC102, IC105, IC106, IC107, IC108, IC109, IC10, IC110, IC111, IC112, IC118, IC119, IC112, IC125, IC126, IC128, IC129, IC12, IC130, IC131, IC133, IC134, IC135, IC136, IC13, IC14, IC1598, IC1599, IC1600, IC1601, IC1602, IC1604, IC1605, IC1607, IC1632, IC1633, IC1638, IC1640, IC1642, IC17, IC18, IC1, IC21, IC22, IC23, IC24, IC27, IC29, IC22, IC327, IC32, IC33, IC34, IC35, IC36, IC37, IC39, IC40, IC42, IC484, IC486, IC487, IC489, IC496, IC497, IC502, IC504, IC505, IC506, IC507, IC508, IC509, IC510, IC512, IC514, IC516, IC520, IC521, IC522, IC529, IC52, IC530, IC532, IC533, IC53, IC540, IC541, IC542, IC543, IC544, IC545, IC548, IC549, IC54, IC554, IC555, IC55, IC5, IC68, IC7, IC807, IC814, IC81, IC83, IC844, IC84, IC853, IC86, IC88, IC8, IC921, IC95, IC97, IC99
H2	IC448
H3	IC1028, IC1056, IC1065, IC1089, IC1103, IC1104, IC1230, IC1233, IC1249, IC1251, IC1260, IC285, IC406, IC650
H4	IC1061
H5	IC329, IC330
H6	IC806

Table S3.2. Continued

H7	IC517, IC518
H8	IC732
H9	IC832, IC848
H10	IC906, IC907, IC920
H11	IC811, IC813, IC836, IC860, IC876
H12	IC331
H13	IC911
H14	IC1142
H15	IC1113, IC1118, IC1119, IC1120, IC1121, IC1133, IC1141
H16	IC1117
H17	IC1134
H18	IC1135
H19	IC458
H20	IC431
H21	IC467
H22	IC1274, IC1291, IC1293
H23	IC1303
H24	IC673
H25	IC402, IC425, IC428, IC432, IC433, IC435, IC437, IC439, IC447, IC449, IC463, IC464, IC465

Table S3.2. Continued

H26	IC310, IC397, IC398, IC401, IC404, IC408, IC414, IC418, IC419, IC423, IC434, IC442, IC445, IC452, IC453, IC456, IC460, IC461, IC469
H27	IC466
H28	IC867
H29	IC1144, IC1146, IC1148, IC1149, IC1150
H30	IC1044, IC1082
H31	IC1053
H32	IC1071
H33	IC1073
H34	IC1072
H35	IC1039, IC1068, IC1070
H36	IC1106
H37	IC1043, IC1045, IC1055, IC671
H38	IC1084, IC1085
H39	IC399
H40	IC1083, IC1272, IC1306
H41	IC1067
H42	IC1033, IC1074, IC1098, IC670
H43	IC1179, IC1239, IC1304, IC309
H44	IC712

Table S3.2. Continued

H45	IC296
H46	IC1280
H47	IC1037, IC1041, IC1059, IC1081, IC289
H48	IC400, IC407, IC412, IC429, IC444
H49	IC1097
H50	IC1257, IC1282, IC1290, IC1296, IC664, IC666, IC667
H51	IC1060, IC1241, IC1271
H52	IC1052, IC1279, IC291, IC648, IC709
H53	IC276
H54	IC490
H55	IC1152
H56	IC679
H57	IC643
H58	IC1048, IC1050
H59	IC1049, IC1305, IC306, IC677, IC711
H60	IC1090
H61	IC227, IC228, IC229, IC232, IC233, IC279, IC286, IC312, IC405, IC662
H62	IC698
H63	IC245, IC248, IC249, IC250, IC251
H64	IC1038, IC1066, IC253

Table S3.2. Continued

H65	IC1254, IC288
H66	IC1094, IC267, IC268, IC269
H67	IC1087
H68	IC1035, IC1036, IC1042, IC1063, IC1091, IC1093, IC1096, IC1099, IC1101, IC1264, IC1266, IC1275, IC1277
H69	IC1258
H70	IC1079, IC1262, IC719
H71	IC1227, IC1265, IC1281, IC283, IC686, IC696
H72	IC1064, IC263, IC264, IC265, IC305, IC462, IC661, IC663
H73	IC837
H74	IC839
H75	IC863
H76	IC477
H77	IC785, IC787
H78	IC791
H79	IC792
H80	IC799
H81	IC798
H82	IC753, IC760
H83	IC770

Table S3.2. Continued

H84	IC478, IC790
H85	IC732
H86	IC723, IC725, IC727, IC728, IC729, IC736, IC737, IC741, IC748, IC749, IC762, IC779, IC780, IC796, IC797
H87	IC751, IC755, IC768, IC777
H88	IC743
H89	IC758
H90	IC742
H91	IC731, IC733, IC735, IC778
H92	IC744
H93	IC720
H94	IC1027, IC1046, IC1047
H95	IC1112
H96	IC1145, IC1147, IC1151
H97	IC1140
H98	IC1029, IC1245, IC274, IC275, IC295, IC678, IC680, IC682
H99	IC1250
H100	IC1153
H101	IC1608
H102	IC262, IC672

Table S3.2. Continued

H103	IC652
H104	IC702
H105	IC1154, IC1156, IC1157, IC1160, IC1161, IC1162, IC1163, IC1164, IC1165, IC1167, IC1168, IC1169, IC1171, IC1174, IC1175, IC1176, IC1178, IC1228
H106	IC308, IC476, IC479
H107	IC217, IC218, IC219, IC220, IC221, IC222, IC223, IC225, IC277, IC457
H108	IC640, IC642, IC651, IC660, IC676, IC683, IC684, IC697, IC703
H109	IC454
H110	IC1155
H111	IC313
H112	IC1270
H113	IC1596, IC1610, IC1614, IC1627, IC1629, IC1634, IC244, IC278, IC292, IC303, IC304, IC420, IC443, IC899
H114	IC1597, IC1603, IC1606, IC1612, IC1618, IC1620, IC1622, IC1623, IC1628, IC1636, IC1637, IC1641
H115	IC258, IC259, IC260, IC261
H116	IC287
H117	IC411, IC416, IC472
H118	IC1086

Table S3.2. Continued

H119	IC1177, IC226
H120	IC396, IC403, IC430, IC438, IC441, IC474
H121	IC307
H122	IC1058
H123	IC1095, IC1100, IC1102
H124	IC1030, IC1229, IC1295, IC1307, IC204, IC234, IC237, IC238, IC239, IC240, IC241, IC242, IC290, IC294, IC297, IC300, IC301, IC409, IC413, IC415, IC424, IC436, IC459, IC656, IC658
H125	IC1062, IC1088, IC1092, IC1237, IC1268, IC243, IC280, IC281, IC293, IC451, IC646, IC655, IC674, IC695, IC701
H126	IC1255
H127	IC1031, IC1276, IC272, IC273, IC299
H128	IC284, IC298, IC410, IC421
H129	IC1032, IC1040, IC1057, IC1252, IC1269, IC203, IC270, IC271, IC302, IC417, IC427, IC440, IC468, IC470, IC471, IC657, IC659, IC675, IC708
H130	IC1034, IC1077, IC282, IC685, IC688, IC704
H131	IC1253, IC1297
H132	IC1078
H133	IC1080
H134	IC1051

Table S3.2. Continued

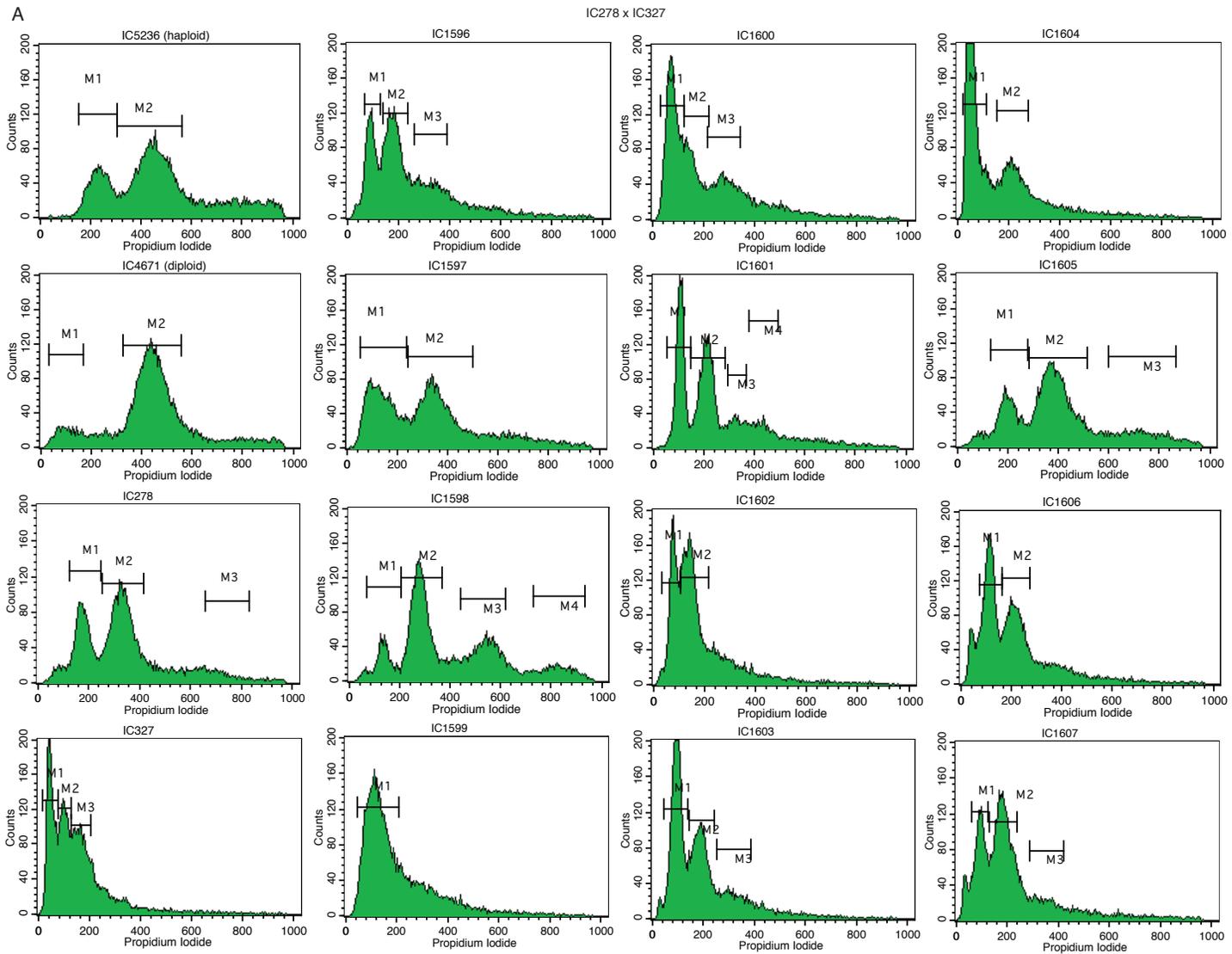
H135	IC1075
H136	IC422
H137	IC446
H138	IC1054
H139	IC426, IC450, IC475
H140	IC1105, IC455
H141	IC1069, IC1076
H142	IC786
H143	IC788
H144	IC851
H145	IC534, IC535
H146	IC875
H147	IC553
H148	IC96
H149	IC494
H150	IC526
H151	IC19
H152	IC672
H153	IC825

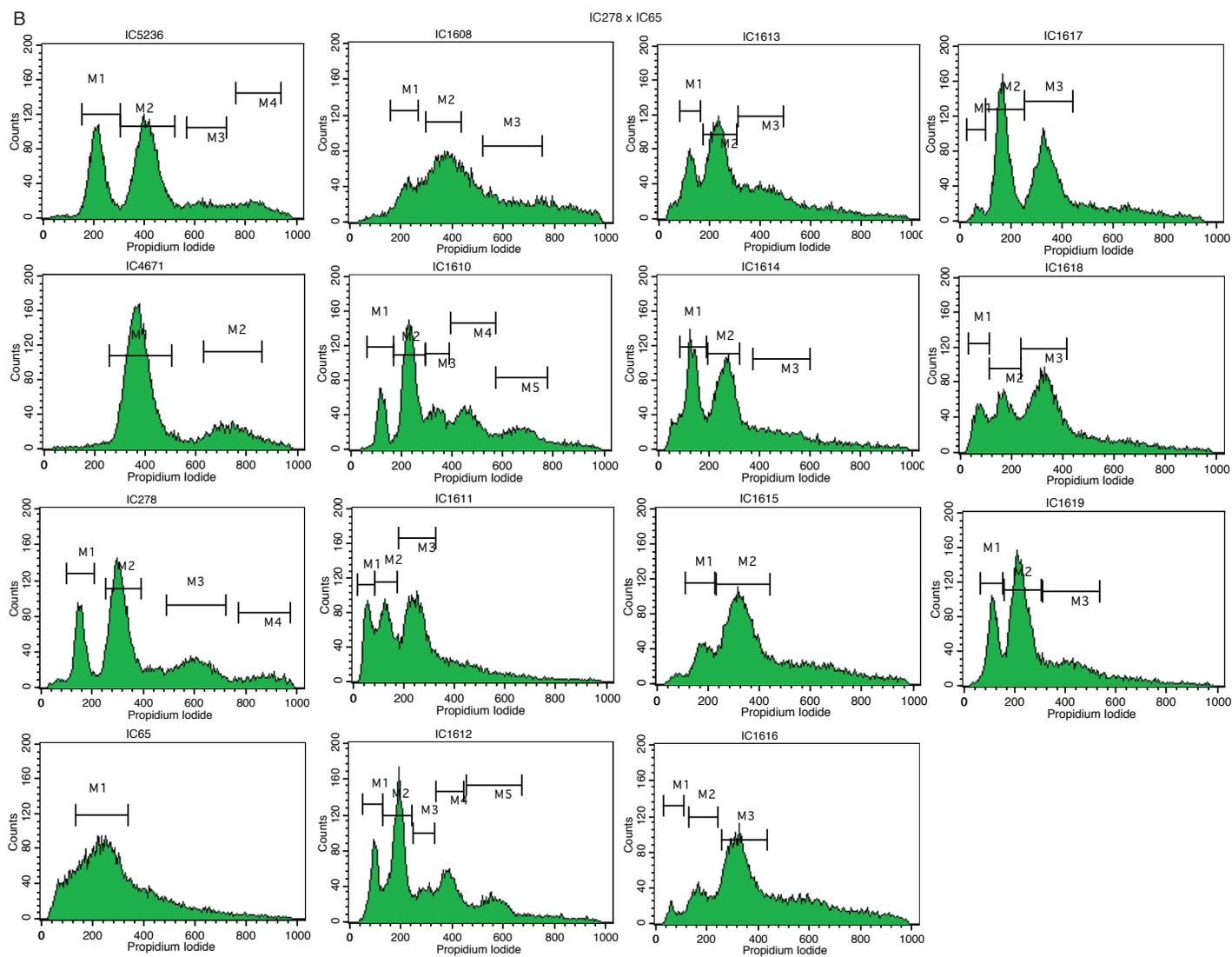
Table S3.2. Continued

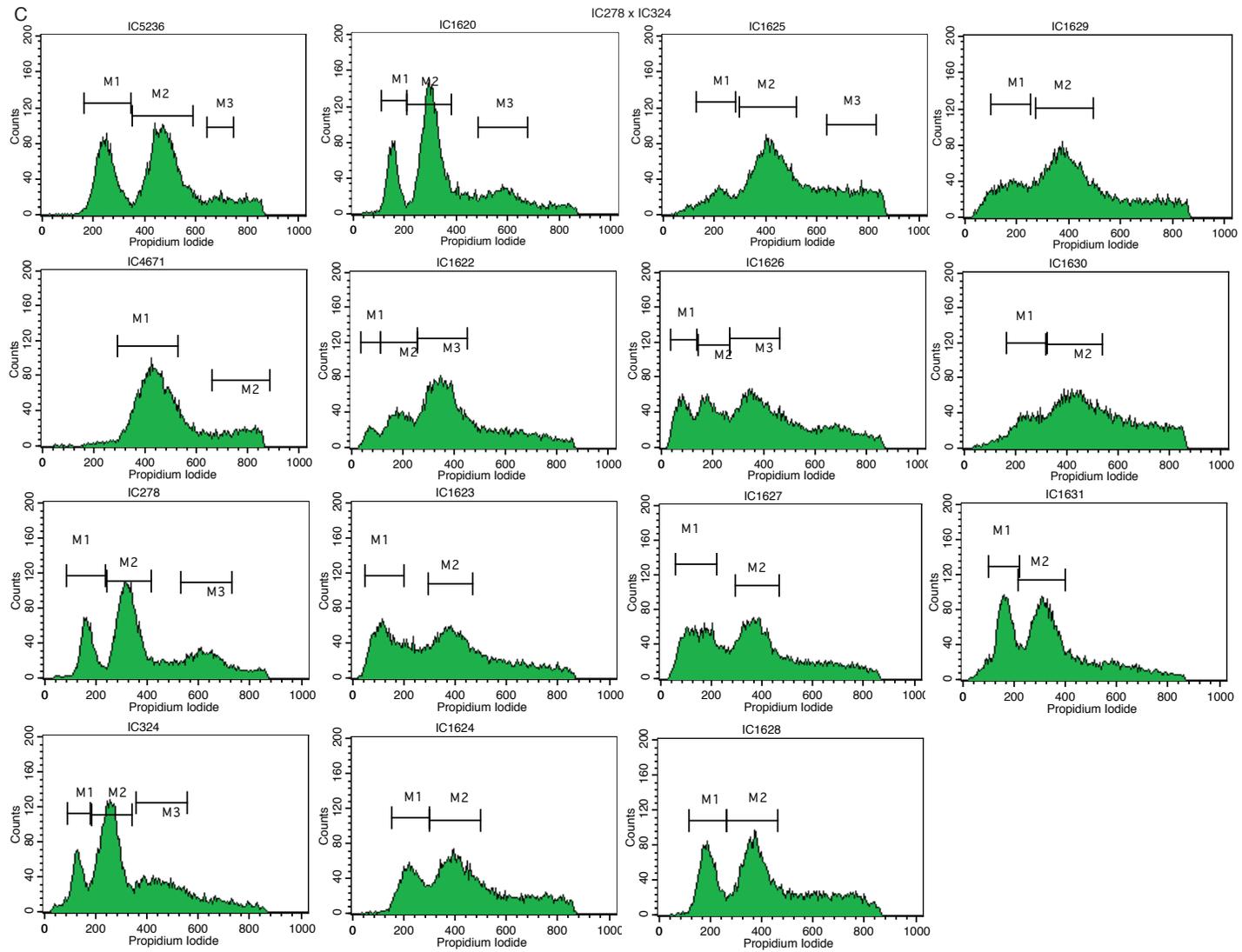
H154	IC1611, IC1615, IC1616, IC1617, IC1626, IC1630, IC317, IC318, IC319, IC320, IC321, IC322, IC323, IC324, IC325, IC326, IC66, IC76, IC809, IC908, IC909, IC910, IC912, IC913, IC915, IC916, IC917, IC918, IC919, IC923, IC924, IC926, IC927
H155	IC1613, IC1619, IC1624, IC1625, IC1631, IC652
H156	IC712
H157	IC1107, IC328, IC58, IC60, IC62, IC63, IC64, IC800, IC801, IC804, IC805, IC808, IC816, IC822, IC828, IC835, IC840, IC854, IC864, IC872
H158	IC824, IC868
H159	IC702
H160	IC742
H161	IC44, IC46, IC47, IC480, IC485, IC48, IC495, IC499, IC49, IC500, IC50, IC511, IC513, IC519, IC51, IC523, IC524, IC525, IC528, IC531, IC536, IC537, IC538, IC539, IC546, IC547, IC551, IC552, IC556, IC905, IC922, IC925
H162	IC1639, IC1643
H163	IC1152, IC252

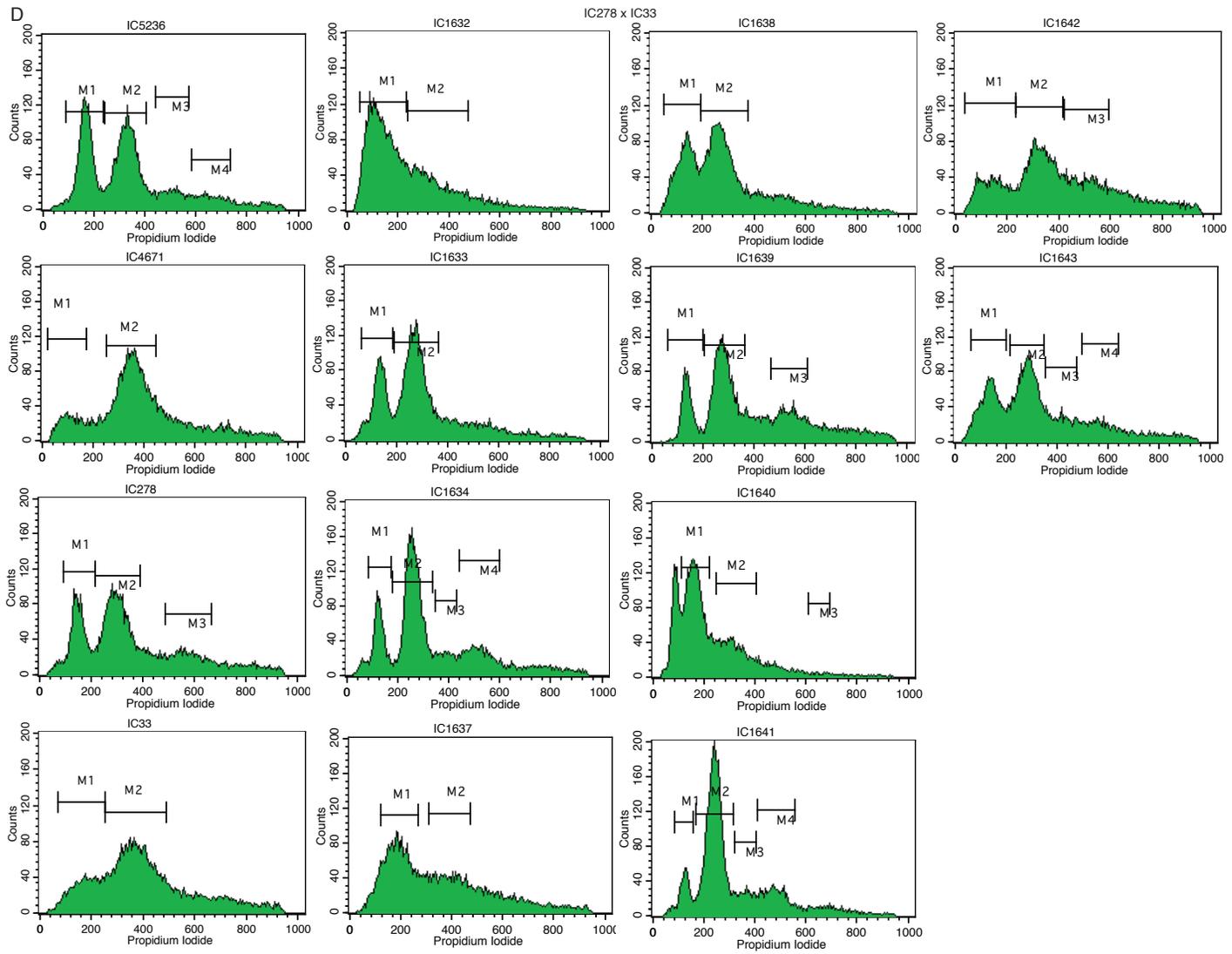
Appendix C

Figure S3.1. Flow cytometric fluorescence histograms of conidia for each strain in the four (A-D) interspecific crosses (Table 3). The histograms show the distribution of intensities (density) of nuclei in conidia stained with propidium iodide; on the x-axis is the intensity of fluorescence and on the y-axis is the number of conidia (counts) at each intensity. Histogram markers are used to specify a range of intensities for populations of conidia at each intensity peak. For example, in the IC5236 haploid histogram in A, the marker M1 is placed around the peak which corresponds to 1N conidia and marker M2 is placed to the right of M1 to designate 1N + 1N conidia.









CHAPTER 4

SEXUAL REPRODUCTION IN *ASPERGILLUS TUBINGENSIS* FROM SECTION *NIGRI*

Short title: Sexual stage of *Aspergillus tubingensis*

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Abstract

A population of a sclerotium-forming member of *Aspergillus* section *Nigri* was sampled from a single field in North Carolina, USA, and identified as *A. tubingensis* based on genealogical concordance analysis. *Aspergillus tubingensis* was shown to be heterothallic, with individual strains containing either a *MAT1-1* or *MAT1-2* mating-type gene. Strains of opposite mating type were crossed on mixed cereal agar and incubated for 5–6 months. Stromata typically formed 1–2 indehiscent ascocarps containing asci and ascospores within the pseudoparenchymatous matrix in a manner similar to the *Petromyces* sexual stage from section *Flavi*, which is closely related to section *Nigri*. Ascospores of *A. tubingensis* differed from those of section *Flavi* species in the reticulate ornamentation of ascospores and the presence of two crests that form an equatorial furrow. Sexual reproduction in *A. tubingensis* may be useful for enhancing enzyme and organic acid production through recombination-mediated genetic engineering of industrial strains.

Introduction

Aspergillus niger and related species, commonly referred to as the ‘black Aspergilli’, are among the most ubiquitous of fungi. Raper and Fennell (1965) placed the species in the ‘*Aspergillus niger* group’ and Gams et al. (1985) formally renamed the group as section *Nigri*. Species from section *Nigri* form a well supported clade within *Aspergillus* based on multilocus DNA sequence analysis (Peterson 2008). Section *Nigri* species are important pathogens of crops and are commonly involved in the deterioration of stored foodstuffs and other organic materials (Raper and Fennell 1965, Kozakiewicz 1989). Several species produce ochratoxin A and fumonisin B₂ (Frisvad et al. 2011), mycotoxins that are of particular concern to the grape and coffee industries (Noonim et al. 2008, 2009; Somma et al. 2012). Section *Nigri* species are used extensively in biotechnology for the production of enzymes (Fogarty 1994, Schuster et al. 2002) and organic acids (Roehr et al. 1992) and for the biosorption of substances from industrial effluent (Grainger et al. 2011) and the bioremediation of contaminated soil (Cortés-Espinosa et al. 2006, Ousmanova and Parker 2007).

Morphology is of limited value for delimiting species within section *Nigri* and molecular methods are often required for identification (Samson et al. 2004, 2007b; de Vries et al. 2005; Noonim et al. 2008; Varga et al. 2011). Hence, many earlier identifications of *A. niger* in the literature are incorrect and represent other closely related species within the ‘*A. niger* aggregate’ (Al-Musallam 1980). Frisvad et al. (2011) reexamined section *Nigri* strains used in industry and found that *A. niger sensu stricto* was the most common species followed by *A. tubingensis*; four other species (*A. acidus*, *A. carbonarius*, *A. brasiliensis* and *A.*

vadensis Samson, de Vries, Frisvad & Visser) were infrequently identified. Ochratoxin A production occurred primarily in *A. niger* and *A. carbonarius* and fumonisin B₂ production occurred only in *A. niger*. Neither mycotoxin was detected in the 83 strains of *A. tubingensis* examined. Therefore, *A. tubingensis* along with several other nontoxigenic species from section *Nigri* were recommended for industrial applications.

Species from section *Nigri* were traditionally considered to be strictly asexual in reproduction. However, in recent years sexual reproduction has been discovered in *Aspergillus* species formerly thought to have lost their ability to undergo meiosis (Geiser et al. 1996). These discoveries have been aided by the identification of mating-type genes and the recognition that many *Aspergillus* species are heterothallic. The crossing of sexually compatible strains from heterothallic species under specific culture conditions has resulted in sexual reproduction in *A. flavus* Link, *A. parasiticus* Speare and *A. nomius* Kurtzman, B.W. Horn & Hesselt. from section *Flavi* (Horn et al. 2009a ,b, c, 2011); *A. fumigatus* Fresen. from section *Fumigati* (O’Gorman et al. 2009); and *A. terreus* Thom from section *Terrei* (Arabatzis and Velegraki 2013).

In this research, sclerotial strains of *A. tubingensis* were obtained from a population in a single field in North Carolina, USA. Following the identification of mating-type genes in this heterothallic species, strains of the opposite mating type were crossed in an attempt to induce sexual reproduction. The sexual stage of *A. tubingensis* is herein described.

Materials and Methods

Aspergillus tubingensis strains.

Isolates were obtained from soil samples collected 14 Sept 2011 from a 73 × 38-m field at the Upper Coastal Plain Research Station, 12 km southeast of Rocky Mount, Edgecombe Co., North Carolina, USA (35°53'58"N 77°40'32"W). The field was in fallow during the year of soil collection and was planted in corn in 2010; soil consisted of Norfolk loamy sand. Twenty soil samples (approximately 150 g each) were collected at 4-m intervals along a diagonal line between opposing corners of the field. Soil samples were air dried and thoroughly mixed, and 33 g soil from each sample was suspended in 100 mL 0.2% water agar. Ten mL of each soil suspension was diluted with 90 mL water agar, and 0.2 mL was then spread on each of ten plates of modified dichloran-rose bengal agar medium (Horn and Dorner 1998). Plates were incubated for three days at 37 C. Colonies of a sclerotial member of section *Nigri* (subsequently identified as *A. tubingensis*) were recognized on soil dilution plates by the presence of dark brown conidial heads and large white sclerotia. Thirty-four strains were randomly selected for mating-type determination and single spored and were further identified on Czapek agar (CZ) (Raper and Fennell 1965) by their aculeate conidia 3.2–4.3 µm in diameter. Population densities of total section *Nigri* and sclerotial *A. tubingensis* were calculated on a dry-weight soil basis.

DNA methods.

Strains of section *Nigri* species used in phylogenetic analyses (Table 4.1) were grown in 25 mL of 2% malt extract broth in 125-mL Erlenmeyer flasks shaken at 200 rpm (25–28 C).

Table 4.I. Species, source of strains, and sequences

Species	NRRL strain	Source	GenBank accession numbers					
			ITS	<i>BT2</i>	<i>CF</i>	<i>Mcm7</i>	<i>RPB2</i>	<i>Tsr1</i>
<i>Aspergillus tubingensis</i> Mosseray	4875 ^T	Unknown	EF661193*	EF661086*	EF661151*	KC796406	EF661055*	KC796442
	4866	Unknown	KC796387	KC796363	KC796379	KC796405	KC796422	KC796441
	62638	Soil, North Carolina, USA	KC796392	KC796364	KC796380	KC796415	KC796431	KC796451
	62639	Soil, North Carolina, USA	KC796393	KC796365	KC796381	KC796416	KC796432	KC796452
	62640	Soil, North Carolina, USA	KC796394	KC796366	KC796382	KC796417	KC796433	KC796453
	62641	Soil, North Carolina, USA	KC796395	KC796367	KC796383	KC796418	KC796434	KC796454
	62642	Soil, North Carolina, USA	KC796396	KC796368	KC796384	KC796419	KC796435	KC796455
	62643	Soil, North Carolina, USA	KC796397	KC796369	KC796385	KC796420	KC796436	KC796456
	62644	Soil, North Carolina, USA	KC796398	KC796370	KC796386	KC796421	KC796437	KC796457
<i>A. acidus</i> Kozak.	4750 ^T	Unknown, Japan	EF661192*	EF661087*	EF661152*	KC796404	EF661052*	KC796440
<i>A. awamori</i> Nakaz.	4951 ^T	Unknown, Brazil	KC796388	KC796358	KC796371	KC796407	KC796423	KC796443
	35710	Peanut seed, Georgia, USA	KC796391	KC796357	KC796374	KC796410	KC796426	KC796446
<i>A. brasiliensis</i> Varga, Frisvad & Samson	26651 ^T	Soil, Brazil	KC796389	KC796355	KC796372	KC796408	KC796424	KC796444
	35574	Peanut seed, Georgia, USA	KC796390	KC796356	KC796373	KC796409	KC796425	KC796445
<i>A. carbonarius</i> (Bainier) Thom	369 ^T	Paper, unknown	EF661204*	EF661099*	EF661167*	JQ689995*	EF661068*	KC796438
<i>A. eucalypticola</i> Varga, Frisvad & Samson	62632 ^T	<i>Eucalyptus</i> leaves, Australia	KC796400	KC796360	KC796376	KC796412	KC796428	KC796448
<i>A. neoniger</i> Varga, Frisvad & Samson	62634 ^T	<i>Verongia</i> sp, Venezuela	KC796401	KC796361	KC796377	KC796413	KC796429	KC796449
<i>A. niger</i> Tiegh.	326 ^T	Tannic acid fermentation, Connecticut, USA	EF661186*	EF661089*	EF661154*	KC796403	EF661058*	KC796439
	62637	Beans, Indonesia; ex type <i>A. lacticoffeatus</i> Frisvad & Samson	KC796402	KC796362	KC796378	KC796414	KC796430	KC796450
<i>A. piperis</i> Samson & Frisvad	62631 ^T	Black pepper, Denmark	KC796399	KC796359	KC796375	KC796411	KC796427	KC796447

^TType strain.

*Sequences retrieved from GenBank database.

Mycelium was harvested after 1–2 days growth by vacuum filtration over Whatman #1 filter paper and then placed loosely in microfuge tubes, frozen and freeze dried. The freeze-dried biomass was ground to a fine powder and rehydrated with 0.5 mL CTAB buffer (Soares et al. 2012). Proteins were extracted by the addition of 0.5 mL chloroform. After brief emulsification, the aqueous phase was separated from the organic phase by centrifugation. The aqueous phase was transferred to a clean tube and DNA was precipitated by the addition of 0.5 mL isopropanol. The precipitate was collected by centrifugation and rinsed with 70% ethanol. The pellet was rehydrated with 0.1 mL TE buffer and stored at -20 C until used.

Beta tubulin (*BT2*), calmodulin (*CF*), nuclear internal transcribed spacer region (ITS), DNA replication licensing protein (*Mcm7*), RNA polymerase II (*RPB2*) and ribosome biogenesis protein (*Tsr1*) loci were amplified from 1:10 diluted genomic DNA using previously described primers and conditions (Soares et al. 2012). Amplified DNA was prepared for sequencing reactions with ExoSapit (<http://www.affymetrix.com>). Sequencing reactions were performed using DyeDeoxy terminator (v3) kits following the manufacturer's instructions followed by analysis on an ABI 3730 DNA analyzer (<http://www.appliedbiosystems.com>). Sequencing was performed in both directions, and any conflicts were resolved using Sequencher 5 (<http://www.genecodes.com>) to visualize the sequences. Sequence discrepancies were found between our sequences and the GenBank deposited sequences of several ex type strains (*A. neoniger* NRRL 62634, *CF* locus FJ491700; *A. piperis* NRRL 62631, *BT2* locus EU163267 and ITS locus DQ900603; *A. eucalypticola* NRRL 62632, *CF* locus EU482433; and *A. lacticoffeatus* NRRL 62637, *BT2* locus DQ900604). Sequences were carefully reviewed for quality and experiments were

repeated if there was doubt about the reliability of sequence reads. DNA sequences were deposited in GenBank (Table 4.1).

DNA sequences were aligned using CLUSTAL W (Thompson et al. 1994). Aligned datasets were analyzed using maximum parsimony (PAUP*, Swofford 2003) and 1000 bootstraps. Conditions for parsimony analysis were random sequence addition, 5000 repetitions, and swap=NNI (nearest neighbor interchange), with maximum trees set to 10000. The results were used as a starting point for subsequent parsimony analysis with sequence addition=ASIS and swap=TBR (tree bisection and reconnection). *Aspergillus carbonarius* was used as the outgroup species for the analysis based on the study by Varga et al. (2011). Tree files were visualized using TreeView (Page 1996) and redrawn for publication using CorelDraw X6 (<http://www.corel.com>). Genealogical concordance was assessed through comparison of the individual locus trees. The least inclusive clade (LIC) that included all of the strains used in crosses was marked for each tree. These least inclusive branches were scored concordant if strains formed identical clades that were statistically supported, scored non-contradictory if they had bootstrap support less than 70%, and scored contradictory if the differences were supported by the bootstrap statistic. Majority rule was used to assess species boundaries (Dettman et al. 2003). Alignments were deposited in TreeBase (<http://purl.org/phylo/treebase/phyloids/study/TB2:S14031>).

Determination of mating type.

DNA extraction of 30–50 mg lyophilized mycelium was performed using Qiagen Maxiprep kit (Qiagen, Valencia, California) following the manufacturer's protocol. The DNA

suspension was held at 4 C for polymerase chain reaction (PCR) amplification. To determine the mating type of *A. tubingensis* strains, *MATI-1* primers (M1F_Anig, 5'GGTCATCGCGAATGATGGAG3' and M1R_Anig, 5'CAGCGTGCTTTCAACGCATTC3') were designed in a consensus region based on an alignment of the *A. niger* genomic contig An11c0340 (GenBank, AM270251.1) and the complete coding sequence of the *A. flavus MATI-1* protein-encoding gene (GenBank, EU357934.1; Ramirez-Prado et al. 2008). Degenerate primers were used to amplify *MATI-2* (*MAT5-4* and *MAT3-2*; Rydholm et al. 2007). PCR amplification was performed in 20- μ L reactions, which included 1 μ L of each primer at 10 μ M concentration, 1 U of Red Taq DNA polymerase (Apex), and 2 μ L of the DNA suspension. Reactions were run in a Mastercycler ep gradient S (Brinkmann Instruments) with a profile of 5 min at 95 C; 40 cycles of 30 s at 95 C, 30 s at 55 C for *MATI-2* and 62 C for *MATI-1*, and 1 min at 72 C; and a final 5 min at 72 C. Following PCR, 5 μ L of PCR product was electrophoresed on a gel and stained with ethidium bromide for visualization. DNA sequencing was described previously (Carbone et al. 2007, Moore et al. 2009); DNA sequences were aligned and manually edited using Sequencher Version 4.7 (Gene Codes Corporation, Ann Arbor, Michigan). Homology searches of putative *MATI-1* and *MATI-2* sequences were performed using blastn and tblastx against the NCBI nr database. DNA sequences were deposited in GenBank (submitted, numbers to be added).

Culture conditions and crosses

Single strains and paired strains of the opposite mating type were inoculated onto slants of mixed cereal agar (MCA) (McAlpin and Wicklow 2005) according to Horn et al. (2009b). Preliminary crosses involving two *MATI-2* strains each paired with 15 *MATI-1* strains consisted of five MCA slants per cross and the final six crosses consisted of 25-55 slants per cross (Table 4.2). In addition, three slants of CZ were inoculated with paired strains for each of the six crosses. Slants were incubated at 30 C in darkness for two weeks and then placed in ziplock bags and incubated for an additional 5–6 months. Sclerotia and stromata were harvested, sectioned and examined using light and scanning electron microscopy according to Horn et al. (2009b). Colony characters of *A. tubingensis* were obtained from NRRL 62638–62644, 4875 (ex type) and 4866 grown on CZ and malt extract agar (MEA) (Raper and Fennell 1965) at 25 and 37 C; microscopic characters of the anamorph were determined from colonies on CZ at 25 C. Viability of ascospores was assessed by germination on MEA as described by Horn et al. (2009b).

Results

Soil populations and mating types.

The field contained a soil population density for total *Aspergillus* section *Nigri* of 2400 ± 4103 CFU/g (\pm SD, $n = 20$). The population density for sclerotial strains of *A. tubingensis* was 31 ± 42 CFU/g, which accounted for $4.1 \pm 7.32\%$ of total section *Nigri*. Of the 34 randomly selected *A. tubingensis* strains from the population, two strains were *MATI-2* and the remaining strains were *MATI-1*.

Table 4.2. Incidences of sexual stage in *Aspergillus tubingensis* crosses

NRRL strain ¹		Number of sclerotia/ stromata examined	% with ascospore- bearing ascocarps
<i>MATI-1</i>	<i>MATI-2</i>		
62638		112	0
62639		101	0
62641		113	0
62642		107	0
62644		146	0
	62640	33	0
	62643	13	0
62638	62640	38	86.8
62641	62640	72	93.1
62644	62643	105	94.3
62639	62643	55	94.5
62642	62640 ²	97	94.8
62644	62640	45	100.0

¹Strain numbers (NRRL) from Agricultural Research Service Culture Collection, Peoria, Illinois, USA.

²Dried voucher slant of NRRL 62642 × NRRL 62640 deposited with the National Fungus Collections, Beltsville, Maryland, USA (BPI 892455).

Concordance analysis.

The phylogenetic tree (Fig. 4.1) calculated using maximum parsimony analysis of combined sequence data from *BT2*, *CF*, *Mcm7*, *RPB2* and *Tsr1* loci showed that the least inclusive statistically supported clade that contains all of the strains used in crosses (NRRL 62638–62644) has two subgroups. The individual loci (Fig.4.2) were examined to determine whether those two groups were present in the single locus trees. At the *CF*, *Mcm7* and *Tsr1* loci, the strains from sexual crosses were strongly supported as a single distinct branch. At the *BT2* and *RPB2* loci, the *MAT1-2* strains NRRL 62640 and NRRL 62643 formed a minority branch. Three of the five loci placed all of the strains used in crosses on a single branch and therefore the strains can be considered to belong to a single species.

At four of the five loci, the statistically supported least inclusive clade (LIC) containing strains from crosses also included the ex type of *A. tubingensis* NRRL 4875; only the *Mcm7* locus placed the ex type outside of the LIC (FIG. 2). On the basis of this evidence, strains used in crosses were properly identified as *A. tubingensis*. At three of those five loci (*CF*, *Mcm7* and *RBP2*), *A. neoniger* was excluded from the LIC and is therefore considered a species distinct from the strains used in crosses.

Mating-type sequence analysis.

A portion of the *A. tubingensis* *MAT1-1* alpha domain sequence, which was obtained from the consensus of an alignment of the *MAT1-1* strains NRRL 62638, 62639, 62641, 62642 and 62644, was found to share 97% (118/122) DNA sequence identity to the *A. niger* (CBS 513.88) *MAT* alpha 1 partial mRNA sequence (GenBank, XM_001394939.2), and 92%

Figure 4.1. Phylogenetic tree calculated using maximum parsimony and combined sequence data from the *BT2*, *CF*, *Mcm7*, *RPB2* and *Tsr1* loci. Bootstrap values were calculated from 1000 resamplings and values greater than 70% are indicated by bold internode lines. *Aspergillus tubingensis* strains used in crosses are shown in bold.

BT2, CF, Mcm7, RPB2 and *Tsr1* combined,
 3159 characters, 688 variable, 351 pic;
 CI=0.8624, RC=0.7696;
 one most parsimonious tree

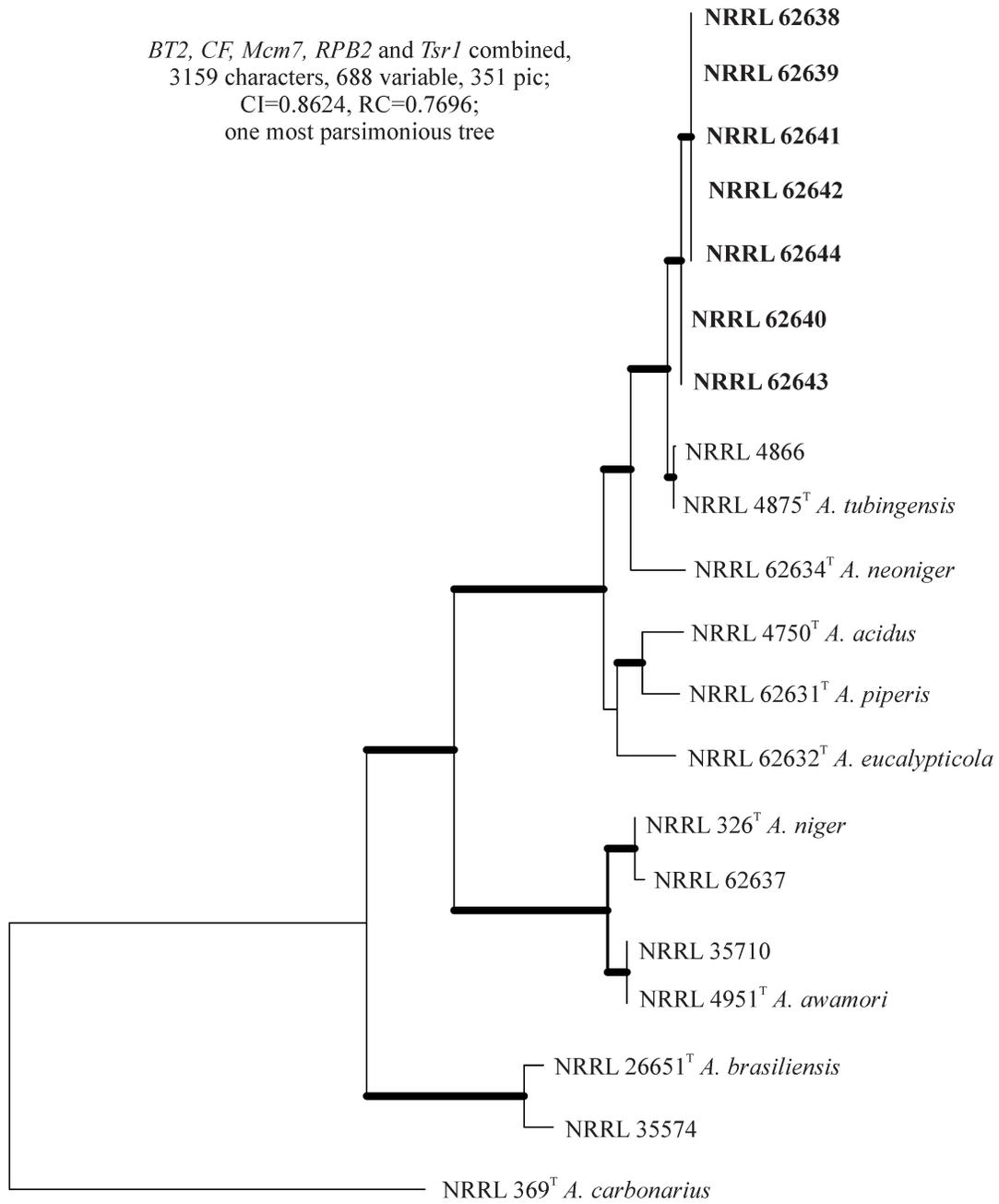
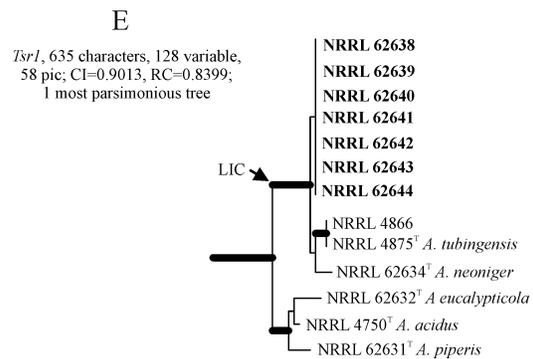
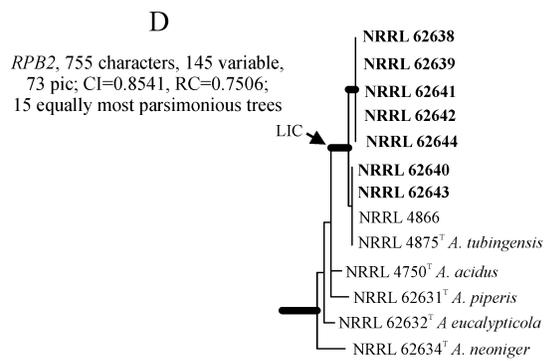
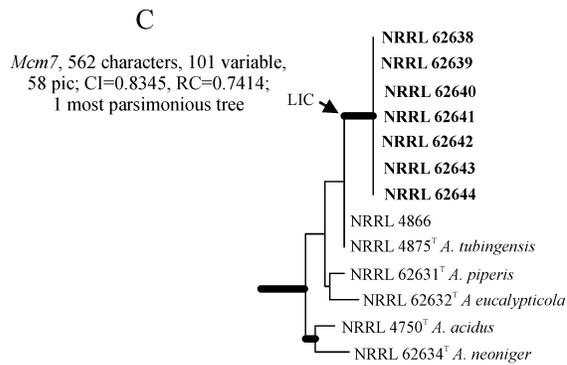
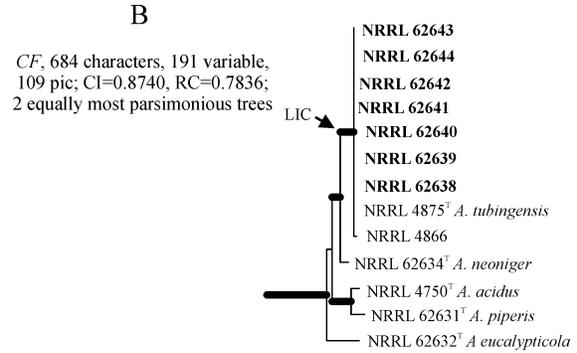
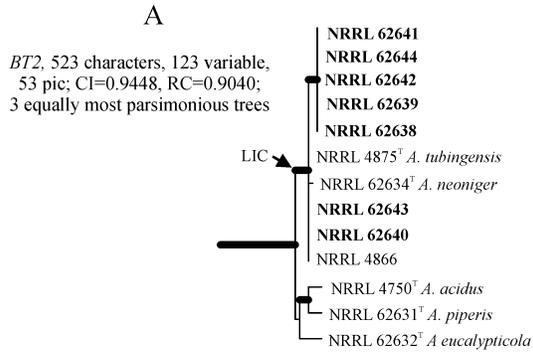


Figure 4.2. Single locus parsimony trees. A. *BT2* locus. B. *CF* locus. C. *Mcm7* locus. D. *RPB2* locus. E. *Tsr1* locus. In each tree, thick branches indicate bootstrap proportions greater than 70% and the least inclusive clade (LIC) that includes all of the strains used in crosses is indicated with an arrowhead.



(390/426) similarity to the *A. niger* genomic contig An11c0340 sequence (GenBank, AM270251.1); the translated *MATI-1* sequence in tblastx searches showed 100% (40/40) and 87% (122/141) amino acid sequence identity, respectively. An intron was identified at positions 256–306 in the *MATI-1* sequence of *A. tubingensis*. The *MATI-2* HMG domain sequence, which was based on the consensus of an alignment of the *MATI-2* strains NRRL 62640 and 62643, did not match anything in the NCBI nr database; however a tblastx search revealed 78–97% amino acid sequence coverage to HMG-box protein-coding sequences in several *Aspergillus* species. The top tblastx hits with highest E-values were to two segments of the *A. oryzae* (GenBank, AB617942.1) and *A. flavus* (GenBank, EU357936.1) *MATI-2* gene, showing 76% (22/29) and 53% (24/45) amino acid sequence identity, respectively. A 5-bp intron was identified at positions 88–92 in the *MATI-2* sequence of *A. tubingensis*.

Sexual reproduction.

The two strains of *A. tubingensis* identified as *MATI-2* (NRRL 62640 and 62643) were each initially crossed with 15 randomly chosen *MATI-1* strains. In the six crosses that produced sclerotia/stromata on MCA (Table 4.2), the number of sclerotia/stromata per slant ranged from 0.8 ± 1.2 (n = 45 slants) in NRRL 62638 \times 62640 to 2.7 ± 3.4 (n = 39) in NRRL 62644 \times 62643. Incidences of ascospore-bearing ascocarps within stromata from the six crosses were high (86.8–100.0%) (Table 4.2). Ascocarps were not produced by any of the single strains. In a preliminary time course on ascocarp development in NRRL 62641 \times 62640 and NRRL 62642 \times 62640 on MCA, small ascocarps without ascospores were observed in the central stromal cavity after 4 weeks incubation in ziplock bags. After 8 weeks incubation,

ascospore-bearing ascocarps had replaced the central cavity and most of the surrounding stromal pseudoparenchymatous matrix (Fig. 4.6). Ascospores from all crosses germinated readily on MEA.

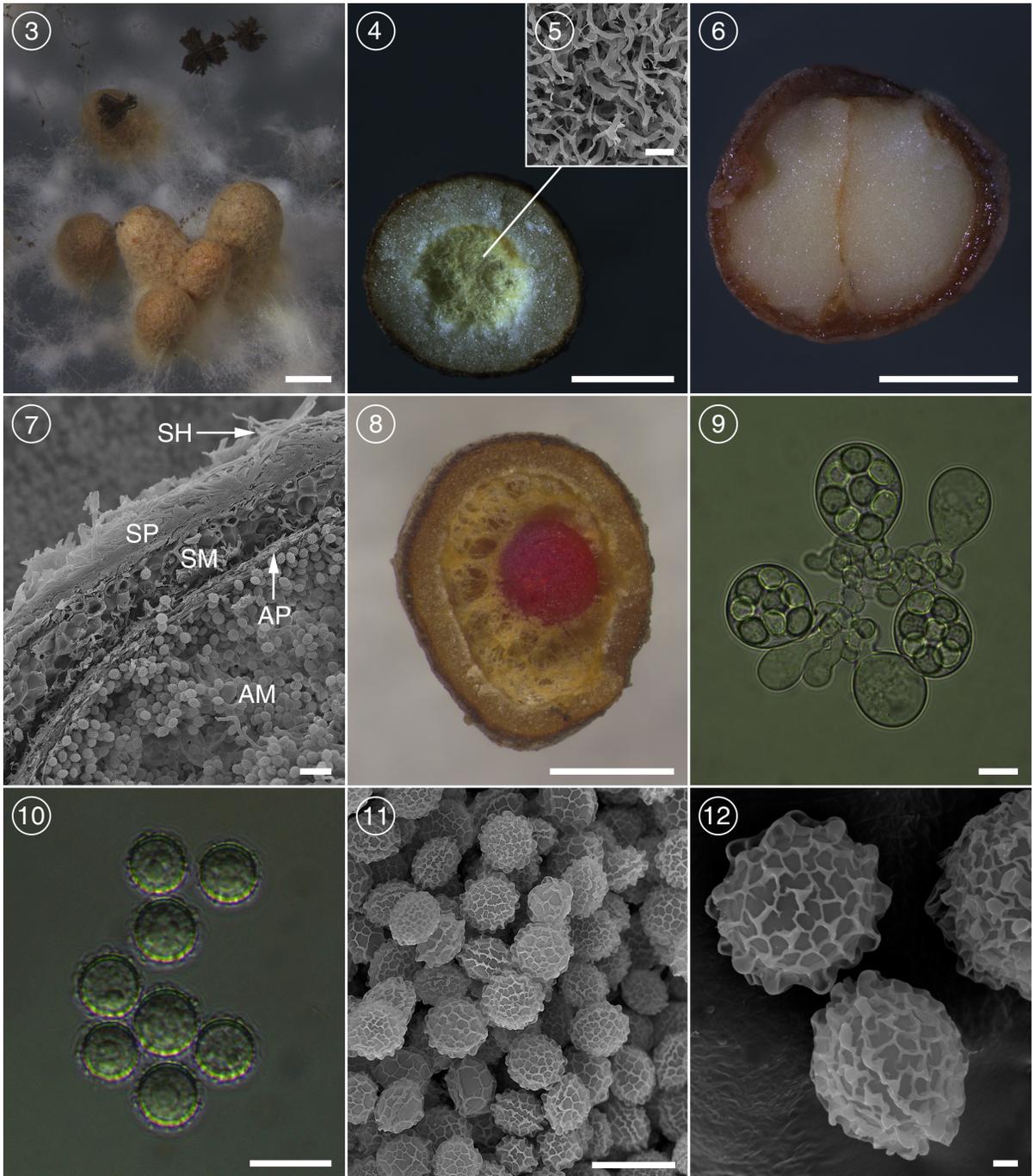
When crosses were performed on CZ slants, sclerotia/stromata were produced in large numbers and stromata usually contained 1–5 small bright orange to orange-red ascocarps within the central cavity (FIG. 8). The incidence of ascocarp formation in stromata on CZ was 78.0–90.2% (n = 8–127) among the six crosses; ascocarps were 150–650 (–800) μm (mean = $344 \pm 130 \mu\text{m}$, n = 69) \times 150–500 (–700) μm (mean = $275 \pm 97 \mu\text{m}$). The ascocarps were mostly filled with undifferentiated hyphae and rarely produced a few ascospores.

Taxonomy

Aspergillus tubingensis Mosseray, La Cellule 43: 245–247 (1934). Figs. 4.3 – 4.12.

Colonies attaining a diameter of 3.5–4.5 cm at 25 C and 7.0–8.5 cm at 37 C after 7 d on Czapek agar and 4.0–6.5 cm at 25 C after 7 d on malt extract agar; generally densely covered with conidial heads; conidial heads en masse at 14 d on Czapek agar grayish brown (7–8G3; Kornerup and Wanscher 1978) or between grayish brown and dark brown (8G3–4); reverse white to cream. *Sclerotia* (Fig. 4.3) and *stromata* similar in external appearance, globose to ellipsoidal, (400–) 500–1600 (–1800) μm (mean = $992 \pm 220 \mu\text{m}$, n = 224) \times (400–) 500–1350 (–1550) μm (mean = $878 \pm 181 \mu\text{m}$), cream to pinkish tan due to outer layer of interwoven filamentous hyphae, with underlying peridium orange brown; matrix pale yellow brown, consisting of pseudoparenchymatous tissue; sclerotia typically with central cavity

Figures 4.3 – 4.13. *Aspergillus tubingensis*. 4.3. Sclerotia formed in culture on Czapek agar. 4.4. Cross section of sclerotium showing cavity filled with loose network of yellow hyphae. 4.5. Hyphae in central cavity of sclerotium. 4.6. Stroma containing two ascocarps. 4.7. Ascocarp peridium (AP) separating a thin layer of the stromal matrix (SM) from the ascocarp matrix (AM) containing ascospores; the stromal peridium (SP) is covered with a layer of filamentous hyphae (SH). 4.8. Sterile ascocarp in central cavity of stroma produced on Czapek agar. 4.9. Asci containing eight ascospores. 4.10. Ascospores. 4.11, 4.12. Ascospores showing lobate-reticulate ornamentation and equatorial furrow flanked by lobate crests. 4.5, 4.7, 4.11, 4.12 = SEM; 4.9 = bright field microscopy; 4.10 = differential interference contrast microscopy. Bars: 4.3, 4.4, 4.6, 4.8 = 500 μm ; 4.7 = 20 μm ; 4.5, 4.9–4.11 = 10 μm ; 4.12 = 1 μm .



containing loose network of light yellow, branched, filamentous hyphae (Figs. 4.4, 4.5). *Ascocarps* (Fig. 4.6) produced within stromata, globose to ellipsoidal but often straight sided where adjacent to other ascocarps, nonostiolate, with white to light brown interior; each stroma containing 1–2 (–4) ascocarps, (250–) 350–900 (–1100) μm (mean = $650 \pm 128 \mu\text{m}$, n = 136) \times 150–750 (–900) μm (mean = $489 \pm 169 \mu\text{m}$), filling most of stroma except for 26.0–87.0 μm outer layer of pseudoparenchymatous matrix; *ascocarp peridium* (FIG. 7) brownish orange, 4.0–8.5 μm thick. *Asci* (Fig. 4.9) globose to broadly ellipsoidal, containing eight inordinately arranged ascospores, 18.0–23.0 (–26.0) μm (mean = $20.6 \pm 1.5 \mu\text{m}$, n = 60) \times (12.0–) 15.0–20.2 (–22.0) μm (mean = $17.4 \pm 1.9 \mu\text{m}$). *Ascospores* (FIGS. 10–12) oblate, lobate-reticulate with an equatorial furrow flanked by lobate crests, hyaline to pale brown, globose to subglobose in face view, 6.0–8.0 μm (mean = $7.0 \pm 0.6 \mu\text{m}$, n = 60) \times 5.1–7.1 (–7.8) μm (mean = $6.5 \pm 0.6 \mu\text{m}$). *Conidial heads* biseriate, rarely uniseriate, radiate, commonly splitting into columns, up to 700 μm in diameter. *Stipes* 550–2300 μm long, 10–18 μm wide near vesicle, light brown, smooth. *Vesicles* globose to subglobose, (27–) 35–60 μm in diameter. *Metulae* 5.0–8.0 \times 3.0–4.8 μm . *Phialides* 5.5–10.0 \times 3.0–4.7 μm . *Conidia* globose to subglobose, 3.2–4.3 (–4.8) μm , tuberculate to aculeate with ridges.

Voucher specimen consisting of dried slant culture with ascocarp-bearing stromata from *A. tubingensis* NRRL 62642 (*MAT1-1*) crossed with *A. tubingensis* NRRL 62640 (*MAT1-2*) was deposited with the National Fungus Collections, US Department of Agriculture, Beltsville, Maryland, USA (BPI 892455). Both strains were isolated from soil collected 14 Sept 2011 from a field in Edgecombe Co., North Carolina, USA. Living

cultures of the two strains have been deposited in the ARS Culture Collection, Peoria, Illinois, USA.

Additional sexual crosses examined: *MAT1-1* × *MAT1-2* crosses included NRRL 62638 × 62640, NRRL 62641 × 62640, NRRL 62644 × 62643, NRRL 62639 × 62643 and NRRL 62644 × 62640.

Discussion

The statistically supported least inclusive clade containing strains used in crosses included the ex type of *A. tubingensis* at four of the five loci examined. Multiple gene trees from a set of isolates are expected to show some contradictions in the branching patterns. Problems in recreating species trees on the basis of individual gene trees and some of the reasons for the incongruity of gene trees have been previously discussed (Maddison 1997, Galagan et al. 2005). Several methods for analysis of gene trees to arrive at species trees have been developed (Baum and Shaw 1995, Taylor et al. 2000, Dettman et al. 2003). Species-level analyses in *Aspergillus* have been conducted mostly in the framework of either polyphasic taxonomy that uses one or more genes and morphological and extralite data to define species (Samson and Varga 2009, Perrone et al. 2011) or genealogical concordance (Peterson 2008, Jurjevic et al. 2012). Genealogical concordance avoids the subjective judgment in phenotypic analysis and in this study, indicated that the strains used in crosses were *A. tubingensis*. The ITS (nuclear internal transcribed spacer) regions were recently proposed as the bar-code locus for identification of fungi (Schoch et al. 2012) and for that reason ITS

sequences were deposited in GenBank (Table 4.1). However, in some cases the use of ITS and lsu-rDNA sequences for phylogeny in *Aspergillus* have proven misleading (Galagan et al. 2005) and therefore ITS was not included in the phylogenetic analysis.

The formation of indehiscent ascocarps delimited by a peridium within the matrix of a stroma in *A. tubingensis* is similar to the *Petromyces* sexual stage in section *Flavi*, as described for *A. flavus* (Horn et al. 2009a), *A. parasiticus* (Horn et al. 2009b,c), *A. nomius* (Horn et al. 2011) and *A. alliaceus* Thom & Church (Fennell and Warcup 1959). *Petromyces* is a teleomorphic genus that was erected by Malloch and Cain (1972) to accommodate the sexual stage of *A. alliaceus*, a species that was initially placed in the *A. wentii* group (Thom and Raper 1945, Kozakiewicz 1989) or the *A. ochraceus* group (Raper and Fennell 1965) because of its yellow conidia but was later shown to belong to section *Flavi* (Peterson 1995). Differences in the sexual stage between *A. tubingensis* and section *Flavi* species primarily involve ascospore characters. Ascospores of *A. tubingensis* are reticulate and have two parallel crests that form an equatorial furrow, whereas ascospores of section *Flavi* are smooth in *A. alliaceus* (Fennell and Warcup 1959) or tuberculate in *A. flavus*, *A. parasiticus* and *A. nomius* and have a single equatorial crest (Horn et al. 2009a, b, c, 2011). Additional descriptions of sexual stages in section *Nigri* are needed to verify differences between the two sections.

The similarity in the sexual stage between sections *Nigri* and *Flavi* is consistent with their phylogenetic placement as sister clades within *Aspergillus* (Peterson 2008). Other closely related clades within the genus show similarities in their sexual stages. For example, the sexual stage of *A. terreus* from section *Terrei* is similar to the *Fennellia* sexual stage of *A.*

flavipes Thom & Church from section *Flavipedes* (Wiley and Fennell 1973, Arabatzis and Velegraki 2013), and the *Neosartorya* sexual stage of *A. fumigatus* and related species is similar to the *Neocarpenteles* sexual stage of *A. acanthosporus* Udagawa & Takada and the sexual stage of *Dichotomomyces cejpai* (Milko) D. B. Scott, both from section *Clavati* (Udagawa and Uchiyama 2002, Samson et al. 2007a, Varga et al. 2007, O’Gorman et al. 2009). The similarity in sexual reproduction in sections *Nigri* and *Flavi* indicates that the *Petromyces* sexual stage is an ancestral trait and that conidial color is more recently derived. Raper and Fennell (1965) divided *Aspergillus* into groups based primarily on conidial color, which encompasses shades of black in section *Nigri* and ranges from yellow through yellow-green to brown in section *Flavi*. However, conidia of several section *Nigri* species are also various shades of brown (Samson et al. 2004, de Vries et al. 2005, Noonim et al. 2008, Varga et al. 2011), so conidial color as a character for separating the sections is not unequivocal.

Sclerotium production is common among species in section *Nigri* (Raper and Fennell 1965, Samson et al. 2004) and sexual reproduction may be widespread within the section, as suggested by the recent report of a teleomorph in *A. scleroticarbonarius* Noonim, Frisvad, Varga & Samson (Darbyshir et al. 2013). Induction of sexual reproduction of *Aspergillus* species in culture often requires specific media and temperatures (McAlpin and Wicklow 2005, O’Gorman et al. 2009, Arabatzis and Velegraki 2013). The formation of diminutive infertile ascocarps of *A. tubingsensis* on Czapek agar, a defined medium, suggests that this medium might be useful for studying the nutritional requirements for different developmental stages in sexual reproduction.

Sclerotial isolates of *A. tubingensis* used in crosses were obtained from a population in which there was a potential for sexual reproduction, as indicated by the presence of opposite mating types. Because of the cryptic nature of many section *Nigri* species, analysis of populations requires the use of molecular techniques to identify isolates and therefore there are few studies showing evidence of genetic diversity in nature. Pekarek et al. (2006) examined a population of *A. niger* from infected cones of the gymnosperm *Welwitschia mirabilis* Hook in Namibia and found high genetic diversity, with 84% of the isolates comprising unique genotypes. However, there was no conclusive proof that the population consisted of a single species. Olarte et al. (2012) showed that *A. flavus* genotypes in a natural population, as characterized by variation at seven multilocus sequence typing (MLST) loci, are also represented by progeny strains generated through laboratory matings.

The population of *A. tubingensis* sampled from North Carolina was predominantly *MATI-1* (94%) and the paucity of *MATI-2* isolates may be the result of a selective disadvantage in the vegetative propagation of *MATI-2* compared to *MATI-1*. Alternatively, *MATI-2* may be associated with female sterility factors that shift populations to a higher frequency of *MATI-1*. Genotyping of progeny strains derived from *A. tubingensis* sexual crosses will reveal the putative female parent in crosses by tracking the inheritance of mitochondrial markers. In addition, DNA sequencing of the progeny strains to elucidate the recombination landscape of their genomes will be important for enhancing enzyme and organic acid production through recombination-mediated genetic engineering of industrial strains.

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CHAPTER 5

TITLE: SEXUAL RECOMBINATION IN *ASPERGILLUS TUBINGENSIS*

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Abstract

Aspergillus tubingensis is closely related to *A. niger*, which is heavily used for industrial applications. Members within *Aspergillus* section *Nigri* have the potential to produce an array of organic enzymes and citric acid. These products are used in food and drink production and preservation, cosmetic and pharmaceuticals to adjust for pH in creams and gels, and even in photography for processing and developing film. Unfortunately, these fungi are also known to produce several mycotoxins such as fumonisin B₂ and ochratoxin A, which both can negatively impact human health. The fungal strains chosen for such industrial applications are those that produce high levels of organic enzymes and citric acid, but are also non-toxigenic. We recently discovered sex in *A. tubingensis* and herein, we genetically prove that the progeny are products of meiosis. We examined six crosses involving five *MAT1-1* isolates and two *MAT1-2* isolates. We used two loci spanning the genome of *A. tubingensis*, RNA polymerase II (*RPB2*) and beta tubulin (*BTa*) and found a total of 60.87% (42/69) recombinant progeny in our sample set. The discovery of sexual recombination in these fungi can benefit human kind by breeding for desirable characteristics (organic enzyme and citric acid production) and breeding against the non-desirable characteristics (toxigenicity).

Introduction

Many members belonging to *Aspergillus* section *Nigri*, including: *Aspergillus niger*, *A. acidus*, *A. aculeatus*, *A. brasillensis*, *A. carbonarius*, *A. japonicus*, *A. niger*, *A. tubingensis*, and *A. vadensis* are used in biotechnology (Frisvad *et al.*, 2011). Industrial uses of these fungi include extracellular enzyme and citric acid production, which are both important in manufacturing products for human consumption. In particular, the extracellular enzyme amyloglucosidase is used extensively in the glucose syrup and alcohol industries; and several enzymes including pectin esterases, endo- and exo- polygalacturonidases and pectin lyases are used in wine and fruit juice production (Grassin, Fauguenbergue, 1999). Citric acid is main acidulant used in the food and beverage industries and is used in such products as soft drinks, fruit juices, desserts, jams, jellies, candy and wine (Frisvad *et al.*, 2011). Citric acid is also important in the pharmaceutical industry, in textile development, in the construction field and in photography to develop film. These fungi undeniably play an integral role in maintaining and sustaining human existence.

These fungi, however, are also known to produce mycotoxins like fumonisin B₂ and ochratoxin A (OTA), which are both detrimental to human and animal health. Some of the most frequently used *A. niger* strains in industry NRRL 337, 3112 and 3122 were all found to produce both toxins, with OTA being more common (Frisvad *et al.*, 2011). Through the years, selection for non-toxigenic industrial strains that produce a wealth of enzymes and citric acid has been facilitated by mutagenesis, parasexual crossing (Das, Roy, 1978), DNA-mediated transformation (Ballance *et al.*, 1983), and recombination techniques (Berka *et al.*,

1992). All of these methods result in a genetically modified organism, which is still not a widely accepted practice. However, with the discovery of sexual reproduction in members of closely related taxa like *A. flavus* Link, *A. parasiticus* Speare and *A. nomius* Kurtzman, B.W. Horn & Hesselt (Horn *et al.*, 2009a; Horn *et al.*, 2011; Horn *et al.*, 2009b; Horn *et al.*, 2009c); *A. fumigatus* Fresen. (O’Gorman *et al.*, 2009); and *A. terreus* Thom (Arabatzis, Velegraki, 2013), and most recently in *A. tubingensis* (Horn *et al.*, 2013), the opportunities are vast for selection of next generation industrial production strains. Herein, we provide genetic evidence for sexual reproduction in *A. tubingensis*, which represents the first report of sexual recombination in section *Nigri*.

Materials and Methods

Sample set and crosses

Isolates were obtained from soil samples collected at the Upper Coastal Plain Research Station in Rocky Mount, North Carolina, USA. Soil sampling and dilution plating were performed as described by Horn *et al.* 2013. Thirty-four randomly selected single colony cultures of *A. tubingensis* were subjected to mating type determination; to determine the mating type of *A. tubingensis* strains, *MAT1-1* primers (M1F_Anig, 5’GGTCATCGCGAATGATGGAG3’ and M1R_Anig, 5’CAGCGTGCTTTCAACGCATTC3’) were designed from a consensus region based on an alignment of the *A. niger* genomic contig An11c0340 (GenBank, AM270251.1) and the complete coding sequence of the *A. flavus* *MAT1-1* protein-encoding gene (GenBank, EU357934.1; (Ramirez-Prado *et al.*, 2008).

Complementary pairs, as well as single strains, were combined onto slants of mixed cereal agar and were allowed to incubate for 5-6 months (both processes described by Horn *et al.* 2013). Six crosses involving five *MAT1-1* isolates and two *MAT1-2* isolates were further examined in this study (Table 1).

DNA isolation and multilocus sequencing (MLST)

Recombination events were detected by examining MLSTs based on variation at two loci: beta tubulin (*BT2*) and RNA polymerase II (*RPB2*). The *BT2* and *RPB2* loci were chosen because according to Horn *et al.* 2013, sequences differed between *MAT1-1* and *MAT1-2* isolates. Exact chromosomal locations with the *A. tubingensis* genome are unclear, however, the *MAT* locus BLASTs to scaffold 5 of the JGI *A. tubingensis* assembled genome and to *A. niger* CBS 513.88 supercontig An11 (NCBI Reference Sequence NT_166526.1). Likewise, the *BT2* and *RPB2* loci BLASTs to scaffolds 1 and 12 of the JGI *A. tubingensis* assembled genome, respectively; and to *A. niger* CBS 513.88 supercontigs An8 (NCBI Reference Sequence NT_166524.1) and An12 (NCBI Reference Sequence NT_166527.1), respectively.

Total genomic DNA was extracted from freeze-dried mycelia using the Qiagen maxiprep kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Sequences of oligonucleotide primers used in PCR amplifications were described previously (Soares *et al.*, 2012), and the steps for DNA sequencing for multilocus sequence typing (MLST) were described previously as well (Carbone *et al.*, 2007; Moore *et al.*, 2009). DNA sequences for each locus were aligned and manually adjusted using Sequencher Version 4.7 (Gene Codes

Corporation, Ann Arbor, MI); alignments were exported as NEXUS files and imported into SNAP Workbench portal (Monacell, Carbone, 2014). Multiple sequence alignments for each locus were collapsed separately into haplotypes using SNAP Map (Aylor *et al.*, 2006) and combined using SNAP Combine (Aylor *et al.*, 2006) for inference of MLSTs. Collapsing into haplotypes was performed with the options of recoding insertions/deletions (indels) for maximal MLST resolution.

Results

Sexual crosses

As reported previously by Horn *et al.* 2013, the chances of finding ascocarps within stromata from the six crosses in this study were great (86.8 – 100.0%). Stromata contained 1 – 5 ascocarps within the central cavity. Ascocarps were not found in any of the sclerotia from single strains. Stromata selected for single-ascospore cultures were cleaned using the methods of Horn *et al.* 2009; and single ascospores were isolated also using methods previously described (Horn *et al.*, 2009a; Horn *et al.*, 2009b; Horn *et al.*, 2009c). Between 12 and 36 single ascospores were isolated depending on the number of ascocarps found within the selected stroma; 12 ascospores were isolated per ascocarp.

MLSTs

Recombination was found in 50.0% (6/12) of the progeny in the cross between parental

isolates IC5107 and IC5109, 66.7% (8/12) of progeny in cross between parental isolates IC5110 and IC5109, 41.7% (5/12) of progeny in cross between parental isolate IC5111 and IC5109, 77.8% (7/9) of progeny in cross between parental isolates IC5116 and IC5109, 50.5% of progeny in cross between parental isolates IC5108 and IC5115, and 83.3% of progeny in cross between isolates IC5116 and IC5115, for a total of 60.87% (42/69) recombinants in the progeny used for genotyping based on the two loci *RPB2* and *BTa* (Table 5.1). The two loci being linked or unlinked and their position within the genome of *A. tubingensis* is unclear, however any combination of alleles that differ from either parent is indicative of recombination.

Discussion

Plants and animals are bred to purposefully manipulate the species in order to create desired genotypes and phenotypes for specific purposes. This is an ancient practice that is still widely used today. Even though the goal of breeding is to select for desirable genotypes and phenotypes, the chances of maintaining or introducing the undesirable genotype or phenotype are the same. In experimental sexual crosses between toxigenic and non-toxigenic *A. flavus* strains, toxigenic progeny were shown to possess the genetic background of the non-toxigenic strain (Olarie *et al.*, 2012). The non-toxigenic parental strain possesses a mutation in an early aflatoxin pathway gene (*aflC*) and a simple crossover event within the aflatoxin cluster, which is located at the distal right end of chromosome 3 is all that is needed to occur to replace the mutation within the cluster that rendered the cluster non-functional. Indeed, a

Table 5.1. Multilocus genotypes from six *Aspergillus tubingensis* crosses

IC Strain	NRRL Strain	<i>MAT</i>	<i>RPB2</i>	<i>BTa</i>	Recombinant
Parents					
IC5107	62638	1	H1	H1	
IC5109	62640	2	H2	H2	
Progeny					
IC5306			H1	H1	No
IC5307			H1	H1	No
IC5308			H2	H1	Yes
IC5309			H1	H1	No
IC5310			H1	H2	Yes
IC5311			H1	H1	No
IC5312			H2	H2	No
IC5313			H2	H2	No
IC5314			H2	H1	Yes
IC5315			H1	H2	Yes
IC5316			H2	H1	Yes
IC5317			H1	H2	Yes
Parents					
IC5110	62641	1	H1	H1	
IC5109	62640	2	H2	H2	
Progeny					
IC5342			H2	H1	Yes
IC5343			H2	H1	Yes
IC5344			H2	H1	Yes
IC5345			H1	H1	No
IC5346			H2	H1	Yes
IC5347			H1	H2	Yes
IC5348			H1	H2	Yes
IC5349			H1	H2	Yes
IC5350			H1	H2	Yes
IC5351			H1	H1	No
IC5352			H2	H2	No
IC5353			H1	H1	No
Parents					
IC5111	62642	1	H1	H1	
IC5109	62640	2	H2	H2	
Progeny					
IC5378			H1	H2	Yes
IC5379			H1	H2	Yes

Table 5.1 continued

IC5380			H2	H2	No
IC5381			H2	H1	Yes
IC5382			H1	H2	Yes
IC5383			H1	H1	No
IC5384			H1	H1	No
IC5385			H1	H2	Yes
IC5386			H2	H2	No
IC5387			H1	H1	No
IC5388			H2	H2	No
IC5389			H1	H1	No
<hr/>					
Parents					
IC5116	62644	1	H1	H1	
IC5109	62640	2	H2	H2	
Progeny					
IC5414			H2	H1	Yes
IC5418			H1	H2	Yes
IC5419			H2	H1	Yes
IC5420			H2	H2	No
IC5421			H2	H2	No
IC5422			H2	H1	Yes
IC5423			H2	H1	Yes
IC5424			H2	H1	Yes
IC5425			H2	H1	Yes
<hr/>					
Parents					
IC5108	62639	1	H1	H1	
IC5115	62643	2	H2	H2	
Progeny					
IC5426			H2	H2	No
IC5427			H1	H2	Yes
IC5428			H1	H1	No
IC5429			H1	H1	No
IC5430			H1	H2	Yes
IC5431			H1	H1	No
IC5432			H1	H2	Yes
IC5433			H1	H1	No
IC5434			H2	H2	No
IC5435			H2	H1	Yes
IC5436			H1	H2	Yes
IC5437			H2	H1	Yes
<hr/>					
Parents					

Table 5.1 continued

IC5116	62644	1	H1	H1	
IC5115	62643	2	H2	H2	
Progeny					
IC5450			H2	H1	Yes
Table 1 continued					
IC5451			H2	H2	No
IC5452			H1	H2	Yes
IC5453			H1	H2	Yes
IC5454			H2	H1	Yes
IC5455			H2	H1	Yes
IC5456			H1	H2	Yes
IC5457			H2	H1	Yes
IC5458			H2	H2	No
IC5459			H1	H2	Yes
IC5460			H2	H1	Yes
IC5461			H1	H2	Yes

progeny isolate was found that possessed the genetic background of its non-toxigenic parent, but recovered its ability to synthesize aflatoxin through a crossover recombination event that reverted the mutation and hence regained full-function of its aflatoxin cluster (Olarde *et al.*, 2012). Recombination through independent assortment of chromosomes is another means of shuffling genetic elements. Despite the potential for progeny to acquire non-desirable traits after genetic recombination, the ability to create a super industrial strain that is also non-toxigenic through natural processes has the potential to revolutionize many industries that rely on the citric acid and enzymes synthesized from members of *Aspergillus* section *Nigri*.

In a previous study we discovered a sclerotium-forming member of *Aspergillus* section *Nigri*, which later proved to be that of *A. tubingensis* (Horn *et al.*, 2013), a very closely related taxa of *A. niger*. The discovery of these sclerotia and the close relatedness between sections *Nigri* and *Flavi* within the phylogeny of the *Aspergilli* prompted experiments to delimit the potential for sexual reproduction within the black *Aspergilli*. Thirty-four strains were randomly selected for mating-type determination and all but two isolates (IC5109 and IC5115) were found to be *MAT1-1*. The disparity between the mating types sampled suggests a prominent asexual lifestyle in nature for *A. tubingensis*. Despite the infrequent occurrence of sex in nature, the potential to have sex still remain, therefore extra care must be taken when handling these fungi, especially those used in biotechnology.

The biosynthetic pathways of ochratoxin A and fumonisin from these fungi are not completely understood, but seem to be associated with multiple genes in the cluster format just like that of aflatoxin in *A. flavus* and *A. parasiticus*. Multiplex PCR and Southern

hybridization analysis suggests that loss of fumonisin B₂ production in *A. awamori* is associated with gene deletions within the fumonisin biosynthetic gene cluster or structural and/or regulatory mutations from elsewhere in the genome that alter gene expression or function (Palumbo *et al.*, 2013). These data are useful in understanding the genetic combinations necessary for identifying non-toxigenic strains for industrial applications. In the present study, 60.9% of the progeny analyzed were found to be recombinants possessing genetic information from both parents. Two loci and six crosses were examined (Table 5.1); in 4 of the crosses, every combination of alleles were produced in the F1 population, which suggests the efficiency of genetic shuffling via sexual reproduction. This method of producing a strain with all desirable traits and no non-desirable traits is preferred over the conventional methods of site directed mutation or genetic transformation because the former is a natural process.

Until only recently, sclerotium production was not known to exist in *A. niger*. Frisvad *et al.* (2013) were successful in inducing sclerotium development from isolates of *A. niger* using a media of white rice and brown rice or adding different fruits to Czapek yeast autolysate agar and incubating at 25 °C. Not only were strains of *A. niger* induced to produce sclerotia, but other species, such as *A. ibericus*, *A. neoniger*, *A. heteromorphus*, *A. figiensis*, *A. luchuensis*, *A. aculeatunius*, and *A. saccharolyticus* were also induced to produce sclerotia on fruit media (Frisvad *et al.*, 2013). Sexual reproduction was also recently found in another member of section *Nigri*, *A. sclerotiicarbonarius* (Darbyshir *et al.*, 2013), which suggests that sex is more common than previously thought; the optimal conditions including media and incubation temperatures are all important for these processes to happen. Given

this information, breeding fungi could very well be the career choice of aspiring young scientists in the future.

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CHAPTER 6

TITLE: POPULATION SHIFTS, MATING-TYPE HETEROKARYOSIS AND
NATURALLY FORMED STROMATA IN *ASPERGILLUS FLAVUS*

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Abstract

Aspergillus flavus is a heterothallic fungal pathogen of many economically important crops worldwide. We sampled *A. flavus* strains from a cornfield in Rocky Mount, North Carolina, USA. Plots were inoculated at tasselling with either *A. flavus* AF36 or NRRL 21882 (=Afla-Guard) nonaflatoxigenic biocontrol strains, both of which are mating type *MAT1-2*. Subsequently, aflatoxigenic strain NRRL 3357 (*MAT1-1*) was applied to all plots, including control plots not inoculated with biocontrol strains. Sclerotia were harvested from infected corn ears and ninety single-ascospore isolates were obtained from ascocarps originating from plots treated with AF36 and NRRL 21882. In addition, eighty *A. flavus* isolates were collected from soil one month after planting (before biocontrol application) and one year after biocontrol application, for a grand-total of 250 isolates. PCR amplification revealed grouping of isolates into three distinct mating-type classes: *MAT1-1*, *MAT1-2* and *MAT1-1/MAT1-2*. An overwhelming majority (54%) of isolates sampled prior to biocontrol treatments were heterokaryotic for mating type (*MAT1-1/MAT1-2*), but was shifted to only 9% of isolates from soil after biocontrol treatments; 39% of isolates obtained from ascospores were heterokaryotic, with the remaining comprising either *MAT1-1* or *MAT1-2*. Multilocus genotyping indicated that ascospores might have originated from Afla-Guard as a putative parent; there was no evidence of AF36 or NRRL 3357 in ascospores or in pre- or post-treatment soil samples, which may explain the genetic structure of the indigenous population. The vertical transmission of *MAT1-1/MAT1-2* to progeny ascospore isolates suggests that heterokaryosis can be maintained in subsequent generations. Furthermore, matings

were performed to determine functionality of these *MAT1-1/MAT1-2* strains and all isolates tested were strictly functional as *MAT1-2*. Further characterization of heterokaryons and their frequency in *A. flavus* populations may be important in understanding the adaptation of these fungi to changing environmental conditions and could lead to better and more effective biocontrol strategies specific to a geographic region. Understanding population structure is the key to unlocking the secrets of a successful biocontrol strain.

Introduction

Recent efforts to reduce aflatoxin concentrations in agricultural commodities have focused on the use of two non-aflatoxigenic (AF-) *Aspergillus flavus* strains, AF36 (=NRRL 18543) and NRRL 21882 (the active component of Afla-Guard). These biocontrol strains are applied at high densities to fields, where they competitively exclude indigenous aflatoxigenic (AF+) strains from crops and thereby reduce AF contamination (Dorner, 2005). AF36 was originally isolated from a cotton field in Arizona and was approved for commercial use on cotton in Arizona and Texas (EPA, 2003). NRRL 21882 was isolated from a peanut seed in Georgia and is currently approved for use on peanuts and corn in the United States (EPA, 2004).

The potential for genetic exchange was not considered when approving these strains for commercial use because sex was not known to exist in these fungi. A cryptic sexual cycle in *A. flavus* was first postulated in 1998 (Geiser *et al.*, 1998). The first account of sexual reproduction in section *Flavi* occurred in 2009 with the discovery of sex in *A. parasiticus* (Horn *et al.*, 2009b; Horn *et al.*, 2009c), and was closely followed by the discoveries of the sexual states in *A. flavus* (Horn *et al.*, 2009a) and *A. nomius* (Horn *et al.*, 2011). The meiotic principles of independent assortment and crossing over were further demonstrated in these fungi through genotyping and array comparative genome hybridization (Olarde *et al.*, 2012). Furthermore, sexual crosses between the non-toxigenic biocontrol strains and toxigenic isolates produced progeny with a regained toxigenic phenotype (Olarde *et al.*, 2012). Sex was also found to increase the diversity of toxin profiles in both

experimental populations (Olarde *et al.*, 2012) and natural population found all over the world (Moore *et al.*, 2013; Moore *et al.*, 2009).

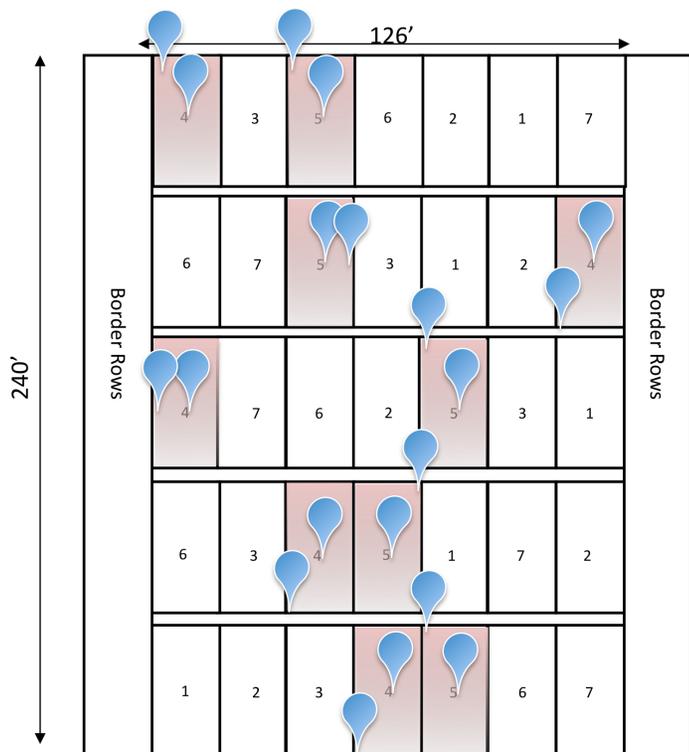
Biocontrol is currently the most effective means of decreasing AF contamination in the short-term; however, usage of biocontrol strains is not sustainable and requires repeat applications yearly to remain effective. One possible explanation is that these strains are disrupting natural population processes, which have a tendency to return to a natural equilibrium in subsequent generations. Quantitative assessments of toxin profiles in agricultural fields spanning several years of biocontrol use have been demonstrated and have shown a significant reduction in toxin loads; however the populations of fungi within a field after biocontrol application has never been examined. In the current research, we aim to examine the population dynamics within a field before and after the application of the biocontrol products.

Materials and Methods

Field site

A cornfield measuring 126 x 240 ft located at the Upper Coastal Plain Station near Rocky Mount, North Carolina, USA was used for this study. The field was planted with corn hybrid DeKalb DKC69-43 at a seeding rate of 28,000 seeds acre⁻¹ and the soil type at this location was a Norfolk loamy sand. The experimental design included a randomized complete block of seven plots each with five replications where the individual plots were six rows wide (12.67') and 40' long, and each block consisted of six plots (Fig. 6.1 – field design).

Figure 6.1. Field design, treatments and soil sampling locations. Treatments included: 1) Check, 2) Afla-Guard (5lbs/acre), 3) Afla-Guard (10lbs/acre), 4) Afla-Guard (20lbs/acre), 5) AF36 (10lbs/acre), 6) A18366 (1.12 Ga/Ha), and 7) A18366 (11.2 Ga/Ha). Treatments 4 and 5 (shaded boxes) were targeted for this experiment because these are the commercial application rates of the respective biocontrol strains. Blue balloons indicate the sampling location of the soil.



- Treatments**
1. CHECK
 2. AflaGuard (5#/acre)
 3. AflaGuard (10#/acre)
 4. AflaGuard (20#/acre)
 5. AF36 (10#/acre)
 6. A18366 (1.12 Ga/Ha)
 7. A18366 (11.2 Ga/Ha)
- *All applied at VT

Soil sampling and fungal isolations

Seven treatments, including a control check, were administered before the corn started to silk (Heiniger, 2011). Soil sampling was confined to the replicate plots of the two treatments that included the commercial application rates of Afla-Guard (20 lbs acre⁻¹) and AF36 (10 lbs acre⁻¹) biocontrol strains. Soil sampling was conducted prior to biocontrol treatment and one year afterward. For each sampling period, two soil samples of approximately 150 g each was collected at each target plot; one sample in the center and another at the periphery of the plot. Soil samples were air-dried and thoroughly mixed, and 33 g soil from each sample was suspended in 100 mL 0.2% water agar. Ten mL of each soil suspension was diluted with 90 mL water agar, and 0.2 mL was then spread on each of ten plates of modified dichloran-rose bengal agar medium (Horn, Dorner, 1998). Plates were incubated for three days at 37 C. Colonies of *A. flavus* were identified on soil dilution plates and transferred onto Czapek agar.

Sclerotia collection, soil incubation and ascospore isolation

Corn ears, including husks, and corn debris were collected from the field. Corn ears were completely shelled by hand and all of the crop debris was examined for the presence of sclerotia. When found, a sclerotium was carefully picked up by tweezers and placed into a 1.5 ml eppendorf tube. Sclerotia were cleaned and allowed to incubate on nonsterile soil for 5 – 7 months as described previously (Horn *et al.*, 2013). Following incubation, sclerotia were cleaned and surface sterilized as previously described (Horn *et al.*, 2013) and cut open to assay for the presence of ascocarps and ascospores. When available, ascospores were

dilution plated onto malt extract agar with antibiotics and examined for germination with the microscope; germlings were then subcultured to CZ plates as described previously (Horn *et al.*, 2009a; Horn *et al.*, 2009c).

DNA isolation and PCR

Total genomic DNA was extracted from freeze-dried mycelia using the Qiagen maxiprep kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The mating type locus and *mfs* locus were used for genotyping; sequence of oligonucleotide primers used in PCR amplifications and DNA sequencing are as described previously (Carbone *et al.*, 2007a; Carbone *et al.*, 2007b; Moore *et al.*, 2009; Olarte *et al.*, 2012). DNA sequences were aligned and manually adjusted using Sequencher Version 4.7 (Gene Codes Corporation, Ann Arbor, MI); alignments were exported as NEXUS files and imported into the SNAP Workbench Portal (Monacell, Carbone, 2014). Sequence alignments were collapsed into haplotypes using SNAP Map (Aylor *et al.* 2006) with the options of recoding insertions/deletions (indels).

Thin Layer Chromatography (TLC)

Presence of mycotoxins (AFs and CPA) was assayed with TLC. Isolates were revived from lyophilized mycelia onto potato dextrose agar (PDA) media and allowed to grow at 30 C for seven days or until sporulation. Spores were transferred using a disposable plastic loop into a 2 ml tube containing 1 ml yeast extract and sucrose (YES) media, the procedure was done in triplicate. Tubes with loosened caps were placed in 30 C incubator for seven days.

Following incubation, 1 ml chloroform was pipetted directly into tube containing growing culture, cap tightened and vortexed for 10 seconds. Tubes containing fungal cultures and chloroform were allowed to sit overnight in fume hood. The following day, 10 µl of chloroform layer was spotted onto TLC plate, along with AF B1, AF G1 and CPA pure extract controls. TLC plates were developed using a 88 : 12 : 0.1 chloroform : acetone : acetic acid solvent and were allowed to run in a sealed container until the solvent front reached approximately one inch from the top of the plate. The plate was then removed from the container and allowed to air dry. The presence of AF was determined by placing the developed TLC plate on a UV box (manufacture) and the presence of CPA was assessed by spraying a solution of 50:50 sulfuric acid : ethanol and a second spray of ethanol and dichlorbenzaldehyde. The TLC plate was then allowed to dry and a purple colored residue was indicative of CPA.

Sexual crosses

Two isolates that were genotyped and found to possess both copies of the mating type gene *MATI-1* and *MATI-2* was crossed with highly fertile compatible *A. flavus* strains and other compatible isolates collected from the same field that were genotyped and found to possess only one copy of the mating type gene, either *MATI-1* or *MATI-2* (see Table 6.3). Each isolate was self-crossed to determine the potential of self-fertility. Crosses were set up following the methods previously described (Carbone *et al.*, 2007a; Carbone *et al.*, 2007b; Horn *et al.*, 2009a; Horn *et al.*, 2009c; Moore *et al.*, 2009; Olarte *et al.*, 2012). For each cross, up to 300 sclerotia, when available, were sliced open and examined for ascocarps and

ascospores. Single ascospore progeny isolates were obtained from individual ascocarps within stromata according to Horn *et al.* 2009b.

Results

Sample set

A total of 80 isolates were obtained in both of the soil sample sets. A total of 90 single ascospore isolates were collected from fertile stromata originating from corn collected in plots in which the Afla-Gaurd and AF36 treatments were deployed. These stromata (Fig. 6.2) were included in the first report of sexual reproduction in *A. flavus* sclerotia naturally produced in corn (Horn *et al.*, 2013). The total number of isolates obtained for this study was 250.

Genotyping

PCR amplification in the mating type locus was successful for 92.6% of the isolates examined. Isolates are grouped into three distinct mating-type classes: *MATI-1*, *MATI-2* and *MATI-1/MATI-2*. The majority (50%) of isolates sampled prior to biocontrol application are heterokaryotic for mating type (*MATI-1/MATI-2*) and is shifted to only 8.75% in the post treatment soil sample set and 32.2% in the post treatment ascospore set (see Table 6.1). The shift in the mating type in the post treatment sets are towards *MATI-2*, making up 83.8% and 45.6% of the post treatment soil and ascospore sample sets, respectively. *MATI-1* remains in the minority comprising 15% of the isolates in the soil sample set prior to biocontrol application, 7.5% of the isolates in the soil sample set post



Figure 6.2. Photo of stroma collected from this field and incubated on soil in the laboratory for 5 – 7 months. A single ascocarp is found in the center of the stroma.

Table 6.1. Mating type classes of sample sets

	Before treatment (soil)	Post treatment (ascospores)	Post treatment (soil)
<i>Mat1-1</i>	12	5	6
<i>Mat1-2</i>	22	41	67
<i>Mat1-1/ Mat1-2</i>	40	29	7
Not determined	6	15	0

biocontrol application and 5.6% of the ascospore isolates.

Genotyping the soil isolates at the *mfs* locus reveals a majority of both sample sets to group into the same haplotype containing the sequence associated with Afla-Guard (Table S6.1). Only one isolate (IC5010) in the post treatment soil set groups together with AF36. The population diversity in the post treatment soil set decreases from eight haplotypes observed prior to biocontrol application to only five in the post treatment set.

Mycotoxin detection

Three mycotoxin classes are detected in the three sample sets, including: AF-/CPA-, AF-/CP+ and AF+/CPA+; AF-/CPA- matches the toxin profile of Afla-Guard (OlarTE *et al.*, 2012) and AF-/CPA+ matches that of AF36 (OlarTE *et al.*, 2012). No isolates are classified as AF+/CPA-. In the soil sample set prior to biocontrol treatment, a fairly equal distribution amongst the three toxin classes is observed (see Table 6.2). In the post treatment soil and ascospore sets, a shift towards the AF-/CPA- toxin class is observed; 86.4% of the total post treatment soil and ascospore sample sets combined are AF-/CPA-, whereas only 12.4% and 1.2% are AF-/CPA+ and AF+/CPA+, respectively.

Sexual crosses

All selfed crosses were unsuccessful in ascospore production, which includes the selfed crosses of the *MATI-1/MATI-2* heterokaryotic individuals (see Table 6.3). Ascospores were only produced in crosses between a *MATI-1/MATI-2* isolate and a previously tested highly fertile *MATI-1* strain (IC278 or IC244) or a *MATI-1* isolate collected from the same field

Table 6.2. Mycotoxin classes of sampling sets

	Before treatment (soil)	Post treatment (ascospores)	Post treatment (soil)
AF-/CPA-	33	77	70
AF-/CPA+	27	12	9
AF+/CPA-	0	0	0
AF+/CPA+	20	1	1

Table 6.3. Incidence of sexual stage in *Aspergillus flavus* crosses

IC number			# sclerotia /stromata examined	% with ascospores	% with aborted ascocarps only	# progeny
<i>MAT1-1</i>	<i>MAT1-2</i>	<i>MAT1-1</i> / <i>MAT1-2</i>				
IC4143			300	0	0	0
IC4142			300	0	0	0
	IC4168		300	0	0	0
	IC4152		152	0	0	0
		IC4197	300	0	0	0
		IC4185	300	0	0	0
IC278		IC4197	300	33.3 ± 2.1	8.3 ± 1.2	36
		IC4185	300	89.0 ± 4.4	1.0 ± 1.0	36
IC244		IC4197	300	25.3 ± 5.7	7.0 ± 1.7	36
		IC4185	300	78.9 ± 4.0	0	36
IC4143		IC4197	300	0	20.7 ± 5.5	0
		IC4185	300	42.0 ± 12.3	0	35
IC4142		IC4197	300	10.3 ± 4.0	17.7 ± 3.8	36
		IC4185	300	87.3 ± 1.2	0	36
	IC202	IC4197	300	0	0	0
		IC4185	300	0	0	0
	IC258	IC4197	300	0	0	0
		IC4185	192	0	0	0
	IC4168	IC4197	300	0	0	0
		IC4185	300	0	0	0
	IC4152	IC4197	300	0	0	0
		IC4185	244	0	0	0

(IC4143 or IC4142). Fertilities ranged from as low as 10.3% to as high as 89.0%. Only one cross (RM-F13 x RM-F67) failed to produce ascospores, however 20.7% of the stromata contained aborted ascocarps, which is the highest percentage among the crosses that produced ascocarps (Table 6.3). Thirty-five to 36 progeny isolates were collected from each cross that successfully produced ascospores.

Discussion

Biocontrol has been widely used around the country for well over a decade, however our study is the first to examine the population structure of the fungal communities before biocontrol application and afterward. We sampled a cornfield over a span of one year, before and after biocontrol application, and we saw that the population diversity was influenced by not only the indigenous population of fungi, but also by the biocontrol used. Stromata formation from naturally formed sclerotia was also witnessed during this study and was previously reported (Horn *et al.*, 2013). Our research is the first of its kind to evaluate these biocontrol products beyond their abilities to reduce toxin levels in agricultural fields. It also examined the impact on the population level of the fungal communities, which furthers our understanding of the mechanisms of these biocontrol strains.

Prior to biocontrol application, a high proportion (54.1%) of the isolates were found to be heterokaryotic for the mating type allele (*MATI-1/MATI-2*) as shown in Table 1. One year after biocontrol application, a shift in the population occurs to favor the *MATI-2* group; this is seen in both the ascospore and soil populations, in which the population density is 54.7% and 83.8%, respectively. This shift may be due to the biocontrol strains AF36 and

Afla-Guard having the *MATI-2* mating type (Moore *et al.*, 2009; Olarte *et al.*, 2012), which suggests that the biocontrol strains are working to replace the indigenous *A. flavus* population. The heterokaryotic mating type profile is carried down to the ascospore population, which indicates heterokaryosis to be a trait that is transferred to progeny after meiosis. This trait has also been seen in experimental populations deriving from sexual crosses between *A. flavus* strains (Olarte *et al.*, 2012).

To determine functionality of the mating type genes in a heterokaryotic individual, mating were performed (see Table 6.3). *MATI-1/MATI-2* isolates were paired with *MATI-1* and *MATI-2* isolates; the homokaryotic individuals tested included isolates used in previous studies that are known to be highly fertile (IC278, IC244, IC202 and IC258) (Horn *et al.*, 2009b; Horn *et al.*, 2009c; Moore *et al.*, 2009; Olarte *et al.*, 2012) or those that were isolated in the same field that were exclusively on a single mating type. Heterkaryotic individuals were not self-fertile, did not produce progeny in crosses involving a *MATI-2* individual and only produced progeny when paired with a *MATI-1* individual, which suggests the heterokaryotic individuals function primarily as the *MATI-2* mating type (see Table 6.3). Further genetic profiling is required to determine the reason why these individuals do not function as *MATI-1*, perhaps a full *MATI-1* allele is not present. The cross between IC4143 (*MATI-1*) and IC4185 (*MATI-1/MATI-2*) produced a fairly abundant number of fertile stromata ($42.0 \pm 12.3\%$), however when IC4143 was paired with IC4197 (*MATI-1/MATI-2*) only aborted ascocarps were produced (see Table 6.3). IC4197 when paired with other strains produces a wealth of ascospore bearing ascocarps and the highest percentage of aborted ascocarps is seen when crossed with IC4197. The female parent in *A. flavus* was

found to be the parent with the *MATI-1* mating type; a reason for the exclusive aborted ascocarp result from crossing IC4143 with IC4197 could involve female sterility, in which IC4143 is the female and IC4197 is the male. The only way progeny can be produced from a sterile female would be by crossing with a highly virulent male partner, which IC4185 could be; when crossed with IC4143, the percentage of sclerotia bearing fertile ascocarps is fair, but is the lowest among the different crosses attempted (see Table 6.3).

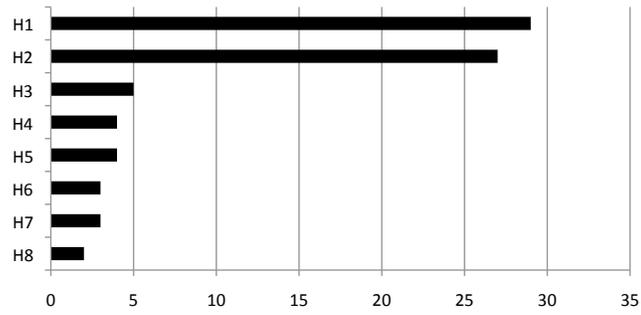
Toxigenic profiles of the isolates indicate a presence of several toxin classes, including AF-/CPA-, which is similar to the toxin profile of the Afla-Guard biocontrol strain, AF-/CPA+, which is characteristic of the AF36 biocontrol strain, and AF+/CPA+. We did not find any AF+/CPA- isolates in our field. Prior to biocontrol treatment, we see a fairly equal distribution between the three toxin classes present; 41% are AF-/CPA-, 33.8% are AF-/CPA+ and 25.0% are AF+/CPA+. One year after biocontrol treatment, a shift in the population favors the AF-/CPA- toxin class, with 85.6% of the ascospores and 87.5% of the soil samples representing this particular toxin class. These data suggest that the Afla-Guard biocontrol strain to be a more successful biocontrol strain.

Further evidence to suggest that Afla-guard is a better biocontrol strain over AF36 in this particular field is in the genotyping of these isolates at the *mfs* locus. The Afla-Guard biocontrol strain groups with the second largest haplotype group in the before-treatment soil set (see Fig. 6.3A). AF36 was not detected in the soil prior to biocontrol application. One year after biocontrol treatment, Afla-Guard groups with the largest haplotype group, which is composed of 87.3% of the total after-treatment soil set (see Fig. 6.3B); AF36 groups with a single isolate from this sample set. In the after-treatment set, the haplotype diversity is

Figure 6.3. Population diversity of soil samples collected before (A) and after (B) biocontrol treatment as established using the *mfs* gene marker. The Afla-Guard biocontrol strain groups into haplotype H2 in A and H1 in B. The AF36 biocontrol strain groups into haplotype H8 in A, which does not contain any soil sample isolates, and into haplotype H5 in B, which contains a single soil sample isolate.

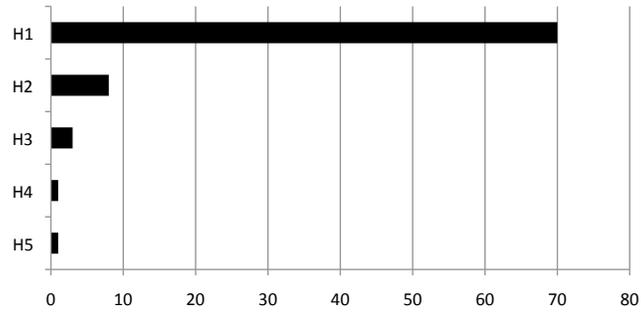
A

Before treatment (soil)



B

After treatment (soil)



decreased, which may be due to the Afla-Guard biocontrol strain dominating the total population. Further genotyping of multiple loci in these samples sets is required to determine the relationship between these isolates and the biocontrol strains.

The success of the Afla-Guard strain in this field may have been predicted by the high abundance of strains found in the same haplotype as Afla-Guard in the before-treatment soil set. This experiment was performed in a field in North Carolina, which is closer to where the Afla-Guard biocontrol strain NRRL 21882 was discovered in comparison to where the AF36 biocontrol strain was discovered. If a similar set of field experiments were conducted in the western part of the country, perhaps the data would favor the AF36 biocontrol strain. Here we suggest that knowing the composition of the indigenous fungal population is important when deciding which biocontrol product to use. The low frequency of AF36-like samples in the post-treatment set may suggest that biocontrols work by increasing the frequency of indigenous non-toxigenic strains. Future research will be directed towards understanding the recombination potential within the ascospore isolates and determine the influence the biocontrols have on directing the population diversity through sexual and/or asexual means.

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APPENDIX

Appendix A

Table S6.1. Haplotypes and strain designations for strains in Figure 6.3

Before treatment (soil)

Haplotype	Strain
H1	IC277, IC4132, IC4135, IC4147, IC4150, IC4151, IC4152, IC4153, IC4159, IC4163, IC4165, IC4169, IC4174, IC4175, IC4176, IC4177, IC4178, IC4183, IC4190, IC4191, IC4192, IC4193, IC4194, IC4197, IC4201, IC4202, IC4206, IC4209, IC4210
H2	IC316, IC4131, IC4133, IC4134, IC4139, IC4140, IC4141, IC4143, IC4144, IC4145, IC4146, IC4160, IC4161, IC4162, IC4166, IC4167, IC4168, IC4170, IC4171, IC4179, IC4181, IC4187, IC4188, IC4199, IC4203, IC4204, IC4207
H3	IC4164, IC4172, IC4180, IC4184, IC4208
H4	IC4136, IC4142, IC4155, IC4158
H5	IC4137, IC4148, IC4185, IC4198
H6	IC4182, IC4200, IC4205
H7	IC4173, IC4195, IC4196
H8	IC1179, IC899

Table S6.1. Continued

After treatment (soil)

Haplotype	Strain
H1	IC316, IC4942, IC4943, IC4944, IC4945, IC4946, IC4947, IC4948, IC4949, IC4950, IC4952, IC4953, IC4954, IC4955, IC4956, IC4957, IC4958, IC4961, IC4962, IC4963, IC4964, IC4965, IC4966, IC4967, IC4968, IC4969, IC4970, IC4971, IC4972, IC4973, IC4974, IC4975, IC4976, IC4977, IC4978, IC4979, IC4981, IC4982, IC4983, IC4984, IC4985, IC4987, IC4989, IC4990, IC4991, IC4992, IC4993, IC4994, IC4996, IC4997, IC4998, IC4999, IC5000, IC5001, IC5003, IC5004, IC5005, IC5007, IC5008, IC5009, IC5011, IC5012, IC5013, IC5014, IC5015, IC5016, IC5017, IC5018, IC5019, IC5020
H2	IC277, IC4941, IC4959, IC4960, IC4986, IC4988, IC5002, IC5006
H4	IC1179, IC899, IC5010
H3	IC4980
H5	IC4951

CHAPTER 7

SEXUAL REPRODUCTION IN *ASPERGILLUS FLAVUS* SCLEROTIA NATURALLY PRODUCED IN CORN

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Abstract

Horn, B. W., Sorensen, R. B., Lamb, M. C., Sobolev, V. S., Olarte, R. A., Worthington, C. J., and Carbone, I. 2013. Sexual reproduction in *Aspergillus flavus* sclerotia naturally produced in corn. *Phytopathology*.

Aspergillus flavus is the major producer of carcinogenic aflatoxins worldwide in crops. Populations of *A. flavus* are characterized by high genetic variation and the source of this variation is likely sexual reproduction. The fungus is heterothallic and laboratory crosses produce ascospore-bearing ascocarps embedded within sclerotia. However, the capacity for sexual reproduction in sclerotia naturally formed in crops has not been examined. Corn was grown for three years under different levels of drought stress at Shellman, Georgia, USA, and sclerotia were recovered from 146 ears (0.6% of ears). Sclerotia of *A. flavus* L strain were dominant in 2010 and 2011 and sclerotia of *A. flavus* S strain were dominant in 2012. The incidence of S strain sclerotia in corn ears increased with decreasing water availability. Ascocarps were not detected in sclerotia at harvest, but incubation of sclerotia on the surface of nonsterile soil in the laboratory resulted in the formation of viable ascospores in *A. flavus* L and S strains and in homothallic *A. alliaceus*. Ascospores were produced by section *Flavi* species in 6.1% of the 6,022 sclerotia (18 of 84 ears) in 2010, 0.1% of the 2,846 sclerotia (3 of 36 ears) in 2011, and 0.5% of the 3,106 sclerotia (5 of 26 ears) in 2012. For sexual reproduction to occur under field conditions, sclerotia may require an additional incubation period on soil following dispersal at crop harvest.

Additional keywords: Aspergillus parasiticus, biological control, Eupenicillium ochrosalmoneum, maize

Introduction

Aflatoxins produced by *Aspergillus flavus* and *A. parasiticus* are among the most potent carcinogens known from nature and also exhibit acutely toxic and immunosuppressive properties (57). These fungi contaminate corn, peanuts, cottonseed, tree nuts and other crops with aflatoxins in the field and during storage (34) and are responsible for major worldwide economic losses due to the costs associated with the monitoring for aflatoxins and the rejection of crops destined for human consumption and animal feed (42,48). Drought and high temperatures during crop development are conducive to invasion by aflatoxigenic fungi and the production of aflatoxins (34). *Aspergillus flavus* is the dominant aflatoxin-producing species in most crops, whereas *A. parasiticus* is most prevalent in peanuts (13).

Aspergillus flavus and *A. parasiticus* belong to *Aspergillus* section *Flavi* (37) and both species commonly produce sclerotia in culture (17). Two morphotypes of *A. flavus* based on sclerotium size have been designated: the L (large) strain, with sclerotia > 400 µm in diameter, and the S (small) strain, with numerous sclerotia < 400 µm (4). Sclerotium formation has been reported in corn by *A. flavus* L strain (51,52), in cottonseed by *A. flavus* S strain (10) and in peanuts by *A. parasiticus* (15). *Aspergillus flavus* sclerotia produced on corn in southern Georgia, USA, were shown to be dispersed onto the soil surface during combine harvesting (52). Dispersal of *A. flavus* sclerotia was accompanied by dispersal of sclerotia of *Eupenicillium ochrosalmoneum*, a homothallic fungus responsible for the production of neurotoxic citreoviridin in corn (53). Sclerotia of *E. ochrosalmoneum* did not contain ascospores at the time of dispersal (52). However, Horn and Wicklow (23) showed

that incubation of *E. ochrosalmoneum* sclerotia from corn on the soil surface results in the formation of ascospores.

Fungal sclerotia are considered to be resistant structures designed to withstand adverse environmental conditions (3). In addition to their role as survival structures, sclerotia of *A. flavus* and *A. parasiticus* germinate sporogenically on soil by producing aerial conidiophores (50,54). It has been postulated that sporogenically germinating sclerotia are a source of primary inoculum in crops (50). However, the role of sclerotia in the life cycle of *A. flavus*, *A. parasiticus* and related *A. nomius* has been recently reassessed with the discovery of the sexual stage associated with these structures (18,19,21,22). *Aspergillus flavus* and *A. parasiticus* are heterothallic and strains typically contain one of two mating-type genes, *MAT1-1* and *MAT1-2* (39). Laboratory crosses between strains of the opposite mating type result in the production of ascospore-bearing ascocarps that are embedded within the matrix of sclerotia. Sexual reproduction in *A. flavus* and *A. parasiticus* produces recombinant progeny through the independent assortment of chromosomes and through crossing over within the aflatoxin gene cluster as well as other portions of the genome (2,22,31,33). Recombination likely accounts for the high genetic variation in field populations of *A. flavus* (33) in which strains range in toxigenicity from nonaflatoxigenic to potent producers of aflatoxins (14,17). Since sclerotia from section *Flavi* act as a repository for sexual structures, they are more appropriately referred to as stromata (22). However, sclerotia lacking a sexual stage are produced by unmated single strains and to varying degrees, by sexually compatible crosses (18,22,33). To avoid confusion in terminology, in

the present paper these structures in section *Flavi* species and *E. ochrosalmoneum* will be called sclerotia regardless of the presence of a sexual stage.

Although sclerotia of *E. ochrosalmoneum* form a sexual stage on soil following dispersal from corn, nothing is known about the capacity of *A. flavus* and *A. parasiticus* sclerotia from crops to produce ascospores. In this study, sclerotia of *A. flavus* were produced in culture from crosses and were harvested before ascocarp formation. Sexual reproduction in these sclerotia was examined under laboratory conditions by incubating sclerotia on the surface of nonsterile soil or when buried in soil. Sclerotia from section *Flavi* also were collected for three years (2010 to 2012) from corn grown in the field under different levels of drought stress. Using the laboratory conditions previously determined to be conducive to ascospore formation, naturally produced sclerotia were incubated on the surface of nonsterile soil to assess their capacity for sexual reproduction.

Materials and Methods

Sexual crosses and sclerotium production.

Aspergillus flavus L strains of the opposite mating type (*MAT1-1* and *MAT1-2*) were crossed to produce sclerotia for comparing ascospore formation under laboratory conditions on culture slants and on the soil surface or when buried in soil. Crosses were performed using highly fertile strain combinations as previously determined (18,33; B. W. Horn, *unpublished data*) (Table 7.1). All crosses involved strains belonging to different vegetative compatibility groups and four of the crosses included commercially available biological control strains NRRL 21882 and AF36 (6). Slants containing mixed cereal agar (MCA) (30) were

Table 7.1. *Aspergillus flavus* crosses for producing sclerotia

<i>MAT1-1</i> ^a				<i>MAT1-2</i> ^a		
Strain ^b	VCG ^c	Aflatoxins ^d		Strain ^{b,e}	VCG ^c	Aflatoxins ^d
29473	17	+	×	29487	25	+
29537	63	+	×	29536	62	+
29507	33	+	×	21882	24	-
29473	17	+	×	21882	24	-
29507	33	+	×	AF36	YV36	-
29473	17	+	×	AF36	YV36	-

^aMating-type designations from Ramirez-Prado et al. (39).

^bStrain number (except AF36) from Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois, USA.

^cVegetative compatibility groups (VCGs) based on Horn and Greene (16), except for YV36 (9).

^dAflatoxin production from Horn et al. (17).

^eBiological control strains are NRRL 21882 (Afla-Guard) and AF36 (= NRRL 18543) (6).

inoculated with conidial suspensions of paired strains and incubated in darkness at 30°C for 2 weeks (22). Sclerotia which had not yet formed ascocarps were removed from slants by scraping in distilled water containing 50 µl/liter Tween 20 and were cleaned by filtering, vortexing in water with glass beads, and rinsing repeatedly with water (22). The air-dried sclerotia were stored in a desiccator jar over saturated NaCl solution at 25°C (75% relative humidity). Additional MCA slant cultures were used for continued incubation at 30°C in sealed plastic bags (22).

Incubation of laboratory-produced sclerotia.

Aspergillus flavus sclerotia on MCA slants and on the surface of soil were examined at the start of the experiment (2 weeks following inoculation of MCA slants, when sclerotia were harvested) and after 4, 8, 12 and 16 weeks additional incubation; sclerotia buried in soil were examined only after 16 weeks. For MCA slant cultures, sclerotia from three replicate slants per cross were harvested at 4-week intervals and 100 sclerotia per slant were dissected with a microscalpel for the presence of ascocarps and ascospores (22).

Soil incubation experiments were conducted using soil with native microbial populations obtained from a cornfield in Shellman, Georgia, USA, where most of the naturally produced sclerotia were obtained (see below). The soil was air dried to $1.6 \pm 0.01\%$ moisture (\pm SD, $n = 3$; dry weight basis) and sieved through No. 12 and 20 Standard Testing Sieves in tandem. Distilled water was added to sieved soil (14 ml per 100 g soil) and the soil was allowed to equilibrate in sealed containers overnight. Moistened soil was added to the rim of 30-cc graduated plastic medicine cups for surface incubation of sclerotia

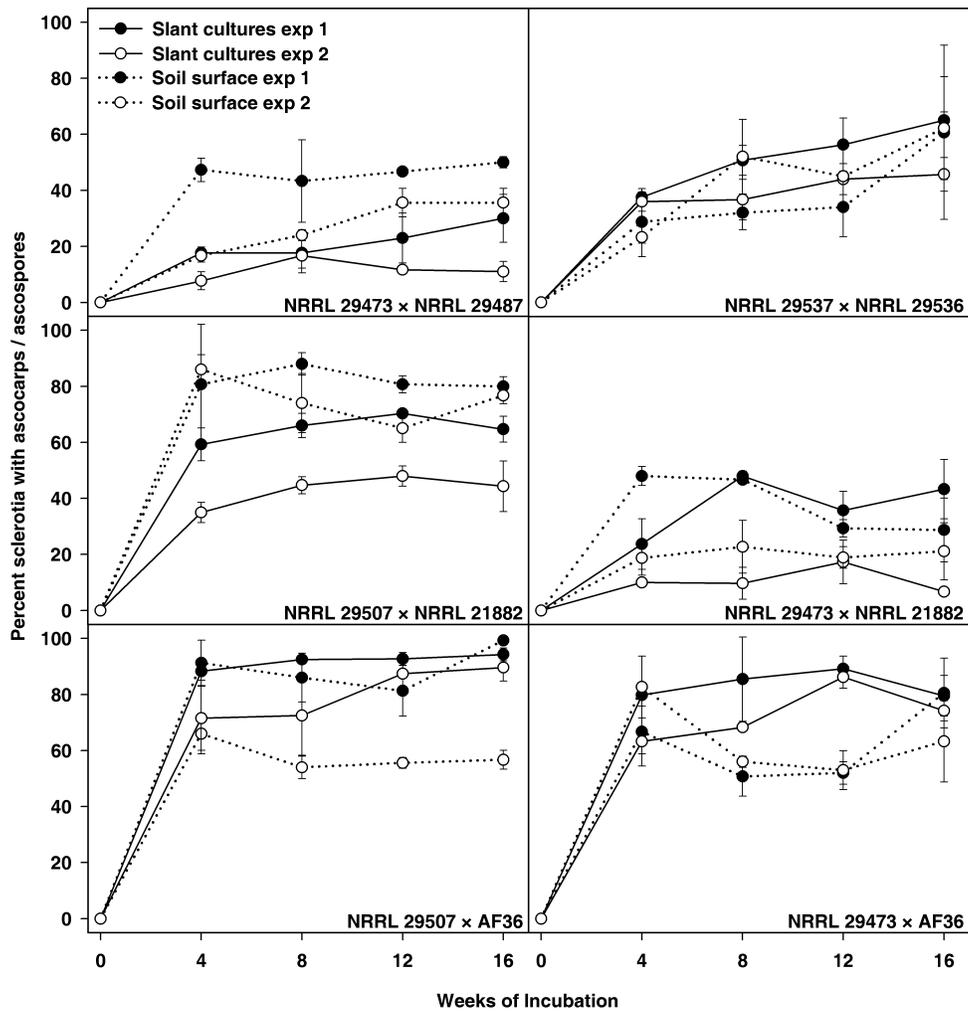
obtained from MCA slants. For buried sclerotia, 25 cc of soil was added to cups, sclerotia were sprinkled on the soil surface, and an additional 5 cc of soil was placed over the sclerotia. Approximately 250 to 350 sclerotia from each cross (Table 7.1) were added to each of three replicate cups. Soil cups were incubated in darkness in a desiccator jar over distilled water at 30°C (100% relative humidity). Fifty sclerotia per cup were randomly removed from the soil surface at each time interval and dissected. Buried sclerotia were retrieved after 16 weeks by wet sieving the top 10 cc of soil in cups through a 100-mesh filter. Viability of buried sclerotia was determined by vortexing sclerotia for 30 s in distilled water with glass beads and surface sterilizing for 2 min with 0.25% sodium hypochlorite followed by several sterile water rinses. Sclerotium halves resulting from the dissection of 20 sclerotia without ascocarps per cup were plated on Czapek agar (CZ) with antibiotics (30 mg/liter streptomycin and 1.5 mg/liter chlortetracycline).

Experiments involving sexual reproduction in laboratory-produced sclerotia on MCA slants, on the soil surface, and buried in soil were performed twice.

Corn cultivation.

Naturally produced sclerotia from section *Flavi* were obtained from corn grown in experimental plots at Shellman, Georgia, in 2010 to 2012 in conjunction with a long-term project by the National Peanut Research Laboratory on water-use efficiency (28). Soil consisted of Greenville fine sandy loam (fine, kaolinitic, thermic Rhodic Kandiudults). Corn was planted and harvested, respectively, on 6 Apr and 12 Aug 2010 (hybrid Pioneer P33M53), 15 Mar and 18 Aug 2011 (Pioneer P1814HR), and 22 Mar and 14 Aug 2012

Figure. 7.1. Ascocarp/ascospore formation in *Aspergillus flavus* sclerotia incubated on mixed cereal agar slants and on the surface of nonsterile soil. Sclerotia were obtained from laboratory crosses. Ascocarps contained asci with mostly immature ascospores after 4 weeks incubation and contained predominantly free mature ascospores at 8 weeks. Datum points represent means \pm SD of three slants or soil cups; results are shown for two experiments.



(Pioneer P1456HR). Corn was grown under conventional tillage in a randomized complete block design consisting of four overhead irrigation treatments with three plots per treatment (28). Each plot consisted of 18 rows of corn planted 0.9 m apart and 30.5 m in length. The treatments provided different levels of drought stress in corn: 0% irrigation (dry land cultivation) and 33%, 66% and 100% of the overhead irrigation rate recommended by Irrigation Pro for Corn software

(<http://www.ars.usda.gov/services/software/download.htm?softwareid=248>). Rainfall and air temperature were recorded from an onsite electronic weather station, and grain yield was determined from the four center rows of each plot (28,45). For measuring aflatoxin concentrations, a 2-kg subsample from the thoroughly mixed grain-yield sample was ground in a Romer Series II Mill (Romer Labs, Inc., Union, Missouri); 200 g of ground corn was extracted and analyzed for aflatoxins by high performance liquid chromatography according to Sobolev and Dorner (44).

Sclerotia from corn.

Preliminary examination of 500 randomly collected corn ears from Shellman indicated that sclerotia were present primarily in ears with visible sporulation by section *Flavi* species; therefore, only ears showing sporulation were hand-harvested for sclerotia. All ears from three randomly selected rows in each of three replicate plots per irrigation treatment were examined at harvest for visible section *Flavi*. In addition to the experimental plots at Shellman, 8 to 32 ears with visible section *Flavi* were collected from four other nonirrigated cornfields in 2010: Field A (1.1 km southeast of Shellman, Randolph Co., Georgia, USA);

Field B (4.8 km northwest of Dawson, Terrell Co., Georgia); Field C (6.4 km south of Shellman, Randolph Co., Georgia); and Field D (12 km southeast of Rocky Mount, Edgecombe Co., North Carolina).

Corn ears were dried at ambient temperature on a forced-air dryer, and kernels from up to three regions with visible section *Flavi* on each ear were dissected and examined externally and internally for sclerotia under the stereomicroscope. Sclerotia from each ear were vortexed for 20 s in 10 ml distilled water without beads, filtered, placed on the surface of nonsterile soil from Shellman in medicine cups, and incubated in darkness in a desiccator jar over distilled water for 5 to 7 months (30°C; 100% relative humidity). Following incubation, sclerotia were cleaned and surface sterilized as previously described for buried sclerotia. Species were identified from 20 sclerotia (when available) by transferring to CZ plates with antibiotics, the sclerotium halves resulting from dissections or in some cases, conidia produced by sclerotia through sporogenic germination or ascospore masses from ascocarps.

Viability of ascospores was determined for individual ascocarps (one ascocarp/sclerotium) from Shellman experimental plots and the additional fields A, C and D in 2010 (105 ascocarps of *A. flavus* L strain and 6 ascocarps of *A. alliaceus*; distributed among 23 ears); from Shellman in 2011 (1 ascocarp of *A. flavus* S strain); and from Shellman in 2012 (3 ascocarps of *A. flavus* S strain and 1 ascocarp of *A. flavus* L strain; distributed among 4 ears). Ascospores were dilution plated onto malt extract agar with antibiotics and examined for germination with the microscope; germlings were then subcultured to CZ plates (22).

Statistics.

All statistics were performed with SigmaStat version 3.5 (Jandel Scientific, San Rafael, California).

Results

Ascospore formation and sporogenic germination in sclerotia from laboratory crosses.

Aspergillus flavus sclerotia were produced on MCA slants using six sexually compatible crosses (Table 7.1). Ascocarp/ascospore formation was compared between sclerotia left on MCA slants and sclerotia harvested from MCA slants before ascocarp formation and then incubated on the surface of nonsterile soil. Although sclerotia did not contain ascocarps at the beginning of the experiments, ascocarps and ascospores formed readily thereafter on both MCA slants and the soil surface (Fig. 7.1). After 4 weeks incubation on MCA slants and soil, ascocarps contained asci with mostly immature ascospores; ascocarps after 8 weeks incubation contained predominantly free mature ascospores following breakdown of the ascus walls. Sclerotia differed in the incidence of ascospore formation according to the specific cross. For example, percent ascospore formation in NRRL 29473 × 29487 and NRRL 29473 × 21882 sclerotia was relatively low ($\leq 50\%$) after 16 weeks incubation on both MCA slants and the soil surface in experiments 1 and 2 and was consistently high ($> 60\%$) in NRRL 29473 × AF36 sclerotia (Fig. 7.1). Percent ascospore formation in NRRL 29473 × 29487 and NRRL 29507 × 21882 sclerotia after 16 weeks was significantly higher (t test; $P < 0.05$; $n = 3$) on soil compared to MCA slants in both experiments. Sclerotia from NRRL 29507 × AF36 were inconsistent in percent ascospore formation between experiments

(significantly lower [$P < 0.001$] on soil compared to slants only in experiment 2); NRRL 29537 × 29536, NRRL 29473 × 21882 and NRRL 29473 × AF36 sclerotia showed no significant differences ($P > 0.05$) in percent ascospore formation between MCA slants and the soil surface in either experiment after 16 weeks.

Sclerotia of *A. flavus* on the soil surface often germinated sporogenically (Fig. 7.4A). Sporogenic germination varied according to the cross. Sclerotia from some crosses were predominantly nonsporogenic (NRRL 29507 × 21882, NRRL 29473 × 21882) and sclerotia from other crosses were lightly sporogenic with only a few conidiophores per sclerotium (NRRL 29473 × 29487, NRRL 29537 × 29536, NRRL 29507 × AF36) or were heavily sporogenic with dense sporulation on the sclerotium surface (NRRL 29473 × AF36). Sporogenic germination did not occur with every sclerotium on the soil surface (Fig. 7.4A). A comparison of ascospore formation in sporogenic and nonsporogenic sclerotia from four crosses showed a significantly (t test; $P < 0.05$; $n = 3$) lower incidence of ascospore formation in sporogenic sclerotia from all crosses in both experiments (Fig. 7.2).

Sclerotia buried in soil for 16 weeks showed reduced ascospore formation compared to sclerotia incubated on the soil surface (Fig. 7.3). Differences were significant (t test; $P < 0.05$; $n = 3$) for sclerotia from all crosses in both experiments, although incidences of ascospore formation in buried sclerotia were often higher in experiment 2. Viability of buried sclerotia without ascocarps among crosses was 1.1 to 31.8% in experiment 1 and 90.0 to 100% in experiment 2.

Figure 7.2. Percent ascospore formation in *Aspergillus flavus* sclerotia with and without sporogenic germination on nonsterile soil after 16 weeks incubation. Sclerotia were obtained from laboratory crosses. Bars represent means \pm SD of three soil cups; results are shown for two experiments.

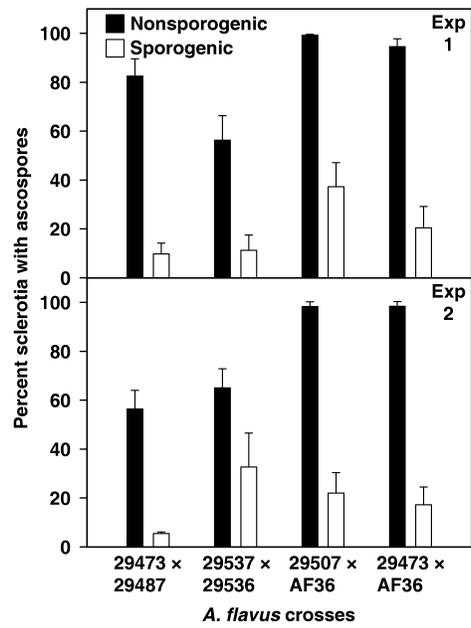
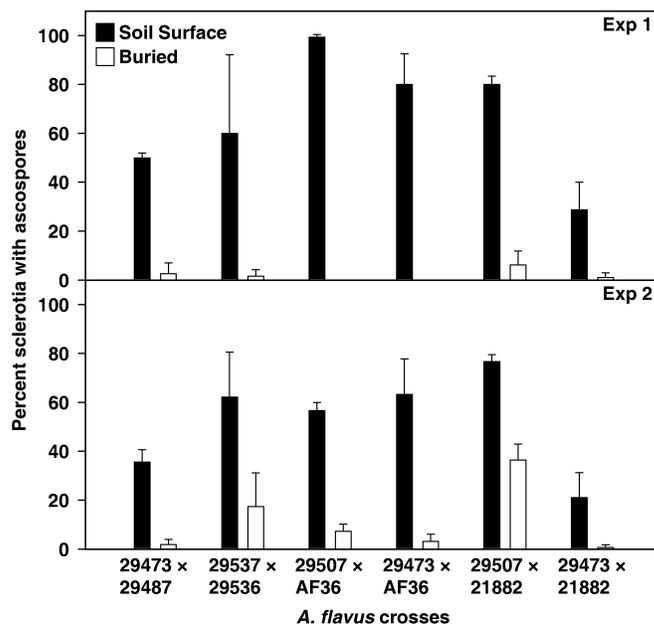


Figure 7.3. Percent ascospore formation in *Aspergillus flavus* sclerotia incubated for 16 weeks on the surface of nonsterile soil or when buried in soil. Sclerotia were obtained from laboratory crosses. Bars represent means \pm SD of three soil cups; results are shown for two experiments.



Effects of drought stress in corn.

Corn was grown in experimental plots at Shellman, Georgia, under different drought stress conditions by supplementing rainfall with varying amounts of water from overhead irrigation (Table 7.2). Average minimum air temperature and average daily temperature were both significantly higher (ANOVA with SNK comparison of means; $P < 0.05$; $n = 30$ or 31) in 2010 compared to 2011 and 2012 for July when corn kernels were in late stages of maturation; average July maximum air temperatures were not significantly different among the three years. The differences in July temperature also were reflected by the higher number of days in 2010 compared to 2011 and 2012 in which the temperature exceeded 37°C (Table 7.2). Rainfall during July was lowest in 2010 for the 3-year period.

The present research was ancillary to the long-term corn project on water-use efficiency at Shellman in which a different corn hybrid was planted for each of the three years. Because of the possible hybrid effect, the effects of water availability on corn yield, aflatoxin concentration and fungal incidence were statistically compared only within individual years. Increasing water availability in corn associated with irrigation led to a significant increase ($P < 0.001$) in grain yield in 2010 ($R^2 = 0.91$; $n = 12$), 2011 ($R^2 = 0.87$; $n = 9$) and 2012 ($R^2 = 0.83$; $n = 12$) (Table 7.3). The 0% irrigation treatment in 2011 did not produce corn ears due to extreme drought conditions in May (0.4 cm rainfall) and June (2.8 cm). Increasing water availability also showed a significant decrease in aflatoxin concentration in 2010 ($R^2 = 0.38$; $P = 0.03$) and 2012 ($R^2 = 0.32$; $P = 0.05$) but not in 2011 ($R^2 = 0.07$; $P = 0.49$). Water availability showed no significant effect ($P > 0.05$) for any of the three years on percentage of ears with section *Flavi* sporulation ($R^2 = 0.17$ to 0.30),

Table 7.2. Temperature, rainfall and irrigation applications during corn ear development at Shellman, Georgia

Year	Month	Minimum air temp (°C) ^b	Maximum air temp (°C) ^b	Average daily temp (°C) ^b	No. days maximum air temp >37 °C	Supplemental irrigation (cm) ^a			
						Rainfall (cm) (0%)	33%	66%	100%
2010	Jun	22.4 ± 1.3	35.1 ± 2.1	27.5 ± 1.9	7	8.5	6.1	12.4	18.5
	Jul	22.9 ± 1.5	35.7 ± 2.8	28.4 ± 1.9	10	8.2	1.7	3.4	5.1
	Aug	22.6 ± 1.3	37.6 ± 5.9	27.7 ± 2.4	9	15.4	0.0	0.0	0.0
2011	Jun	21.5 ± 1.1	36.8 ± 1.7	28.2 ± 1.7	6	2.8	8.5	13.7	18.8
	Jul	22.1 ± 0.8	34.5 ± 2.2	27.1 ± 1.6	2	15.3	1.7	3.4	5.1
	Aug	22.3 ± 1.3	35.8 ± 1.4	27.9 ± 1.2	5	5.4	0.0	0.0	0.0
2012	Jun	19.4 ± 1.9	31.5 ± 3.7	25.0 ± 2.3	2	6.3	5.3	10.8	16.1
	Jul	21.9 ± 0.9	35.3 ± 2.1	27.3 ± 1.5	5	10.8	0.0	0.0	0.0
	Aug	21.1 ± 1.6	31.9 ± 2.2	25.4 ± 1.3	1	5.6	0.0	0.0	0.0

^aPercentage of the overhead irrigation rate recommended by Irrigator Pro for Corn software

(<http://www.ars.usda.gov/services/software/download.htm?softwareid=248>).

^bTemperatures are means ± SD ($n = 26$ to 31) and are based on daily values.

Table 7.3. Yield, aflatoxin concentrations, and sporulation and sclerotium formation by *Aspergillus* section *Flavi* in corn grown at Shellman, Georgia, under different conditions of water availability^a

Irrigation treatment ^b	Grain yield (mt/ha)	Total aflatoxin (ppb)	No. ears examined	No. ears with sect. <i>Flavi</i> sporulation ^c	No. ears with sect. <i>Flavi</i> sclerotia ^c
2010 crop					
0%	1.0 ± 0.1	356.8 ± 163.1	550-814	34-55 (7.3 ± 2.4)	3-14 (1.4 ± 1.0)
33%	7.2 ± 1.1	11.9 ± 17.2	763-801	23-59 (4.3 ± 2.7)	6-9 (0.9 ± 0.2)
66%	11.7 ± 0.9	3.7 ± 4.8	765-795	12-34 (2.8 ± 1.4)	1-11 (0.7 ± 0.7)
100%	13.3 ± 1.4	61.0 ± 64.7	651-681	12-39 (3.8 ± 2.0)	2-11 (0.9 ± 0.7)
2011 crop^d					
33%	0.6 ± 0.4	22.1 ± 35.9	403-513	2-11 (1.3 ± 0.9)	1-4 (0.5 ± 0.3)
66%	3.6 ± 1.8	11.3 ± 9.5	506-710	7-10 (1.3 ± 0.2)	2-4 (0.5 ± 0.2)
100%	9.5 ± 1.9	9.4 ± 11.6	695-721	10-16 (1.8 ± 0.4)	4-8 (0.9 ± 0.3)
2012 crop					
0%	1.6 ± 0.4	78.3 ± 70.6	449-812	8-21 (2.0 ± 0.7)	1-8 (0.6 ± 0.4)
33%	9.4 ± 1.1	0.2 ± 0.4	852-909	3-5 (0.4 ± 0.1)	0-1 (0.04 ± 0.07)
66%	12.6 ± 0.8	0.0 ± 0.0	921-966	3-14 (0.8 ± 0.6)	1-3 (0.2 ± 0.1)
100%	13.3 ± 1.1	3.0 ± 5.2	838-861	3-15 (0.9 ± 0.8)	1-4 (0.2 ± 0.2)

^aMeans ± SD and ranges based on three replicate plots per irrigation treatment.

^bPercentage of the overhead irrigation rate recommended Irrigator Pro for Corn software (<http://www.ars.usda.gov/services/software/download.htm?softwareid=248>).

^cPercentage of total number of ears examined in parentheses.

^d0% irrigation treatment did not produce corn ears.

percentage of ears with section *Flavi* sclerotia ($R^2 = 0.09$ to 0.31), or percentage of ears with *A. flavus* L strain sclerotia ($R^2 = 0.004$ to 0.31) (Tables 7.3 and 7.4).

Sclerotium production in corn.

Dissection of corn ears revealed section *Flavi* sclerotia on the outer kernel surface, inside the kernel when damaged by insects (Fig. 7.4B) and at the base of kernels where attached to the cob. Percentages of ears with section *Flavi* sporulation (0.3 to 10.0%) and sclerotia (0 to 2.5%) in experimental plots were low for all three years (Table 7.3). The majority of sclerotia were produced by *A. flavus* L strain in 2010 and 2011 and by *A. flavus* S strain in 2012 (Table 7.4). *Aspergillus flavus* S strain sclerotia from combined years were detected in 27 ears of which 51.9, 29.6, 18.5 and 0.0% were from the 0, 33, 66 and 100% irrigation treatments, respectively ($R^2 = 0.99$; $P = 0.007$). Sclerotia from other section *Flavi* species included those of *A. parasiticus* (4 ears), *A. alliaceus* (2 ears) and *A. caelatus* (1 ear). The four additional fields from 2010 (Table 7.5) showed a predominance of *A. flavus* L strain sclerotia, with only two ears containing sclerotia of the S strain. Sclerotia of *A. flavus* S strain and *A. parasiticus* frequently co-occurred on the same ear with sclerotia of *A. flavus* L strain (Tables 7.4 and 7.5). Ascocarps and ascospores were not present in *A. flavus* sclerotia ($n = 1226$; distributed among 58 ears) and *A. alliaceus* sclerotia ($n = 11$; distributed among 2 ears) when examined directly from corn ears at harvest.

Ascospore formation in sclerotia from corn.

When section *Flavi* sclerotia collected from Shellman corn were incubated on the surface of

Table 7.4. Formation of ascospores in *Aspergillus* section *Flavi* sclerotia obtained from corn grown in Shellman, Georgia, under different conditions of water availability

Irrigation treatment ^a	No. ears	No. sclerotia incubated ^b	Species ^c	No. sclerotia forming ascospores on the surface of nonsterile soil ^d
2010 crop				
0%	19	1185 (1-565)	F-L	0
	1	176	F-L	30 (17.0)
	1	240	F-L	32 (13.3)
	2	218 (62, 156)	F-L, F-S	0
	1	67	F-L, P	0
	2	334 (124, 210)	F-S	0
	1	1	P	0
	33%	11	296 (1-184)	F-L
	1	39	F-L	1 (2.6)
	1	229	F-L	52 (22.7)
	1	318	F-L	1 (0.3)
	1	58	F-L, F-S	19 F-L (32.8)
	1	274	F-L, F-S	1 F-L (0.4)
	1	500	F-L, F-S	1 F-L (0.2)
	1	6	F-L, F-S	0
	1	33	F-L, P	0
	1	199	F-S	0
	1	21	A	13 (61.9)
	1	105	A	101 (96.2)

Table 7.4. Continued

66%	14	488 (1-137)	F-L	0
	1	26	F-L	9 (34.6)
	1	167	F-L	15 (9.0)
100%	13	261 (1-69)	F-L	0
	1	15	F-L	1 (6.7)
	1	18	F-L	1 (5.6)
	1	95	F-L	22 (23.2)
	1	126	F-L	22 (17.5)
	1	245	F-L	30 (12.2)
	1	282	F-L	16 (5.7)
2011 crop^e				
33%	6	194 (7-60)	F-L	0
	1	204	F-L, F-S	0
	1	391	F-L, F-S, P	1 F-S (0.3)
66%	7	549 (1-300)	F-L	0
	2	489 (243, 246)	F-L, F-S	0
100%	16	890 (3-307)	F-L	0
	1	16	F-L	1 (6.3)
	1	70	F-L	2 (2.9)
	1	43	F-L, C	0
2012 crop				
0%	2	3 (1, 2)	F-L	0
	1	3	F-L	1 (33.3)

Table 7.4. Continued

	1	183	F-L, F-S	4 F-S (2.2)
	1	225	F-L, F-S	3 F-S (1.3)
	2	393 (7, 386)	F-L, F-S	0
	6	611 (8-329)	F-S	0
33%	1	197	F-S	2 (1.0)
66%	3	37 (4-26)	F-L	0
	3	1224 (20-1024)	F-S	0
100%	5	184 (1-83)	F-L	0
	1	46	F-L	7 (15.2)

^aPercentage of the overhead irrigation rate recommended by Irrigator Pro for Corn software (<http://www.ars.usda.gov/services/software/download.htm?softwareid=248>).

^bTotal number of sclerotia; range of sclerotium numbers per ear in parentheses.

^cAbbreviations: F-L, *A. flavus* L strain; F-S, *A. flavus* S strain; P, *A. parasiticus*; C, *A. caelatus*; A, *A. alliaceus*.

^dPercentage of sclerotia producing ascospores in parentheses.

^e0% irrigation treatment did not produce corn ears.

Table 7.5. Formation of ascospores in *Aspergillus* section *Flavi* sclerotia obtained from corn ears in additional fields (2010)

Field	Location	No. Ears	No. sclerotia incubated ^a	Species ^b	No. sclerotia forming ascospores on the surface of nonsterile soil ^c
A	Randolph Co., GA, USA	1	3	F-L	0
		1	72	F-L	3 (4.2)
		1	136	F-L, F-S	3 F-L (2.2)
B	Terrell Co., GA	1	2	F-L	0
C	Randolph Co., GA	2	8 (3, 5)	F-L	0
		1	239	F-L	184 (77.0)
		1	265	F-L, F-S	0
D	Edgecombe Co., NC	8	253 (1-86)	F-L	0
		1	1	F-L	1 (100.0)
		1	216	F-L	7 (3.2)

^aTotal number of sclerotia; range of sclerotium numbers per ear in parentheses.

^bAbbreviations: F-L, *A. flavus* L strain; F-S, *A. flavus* S strain.

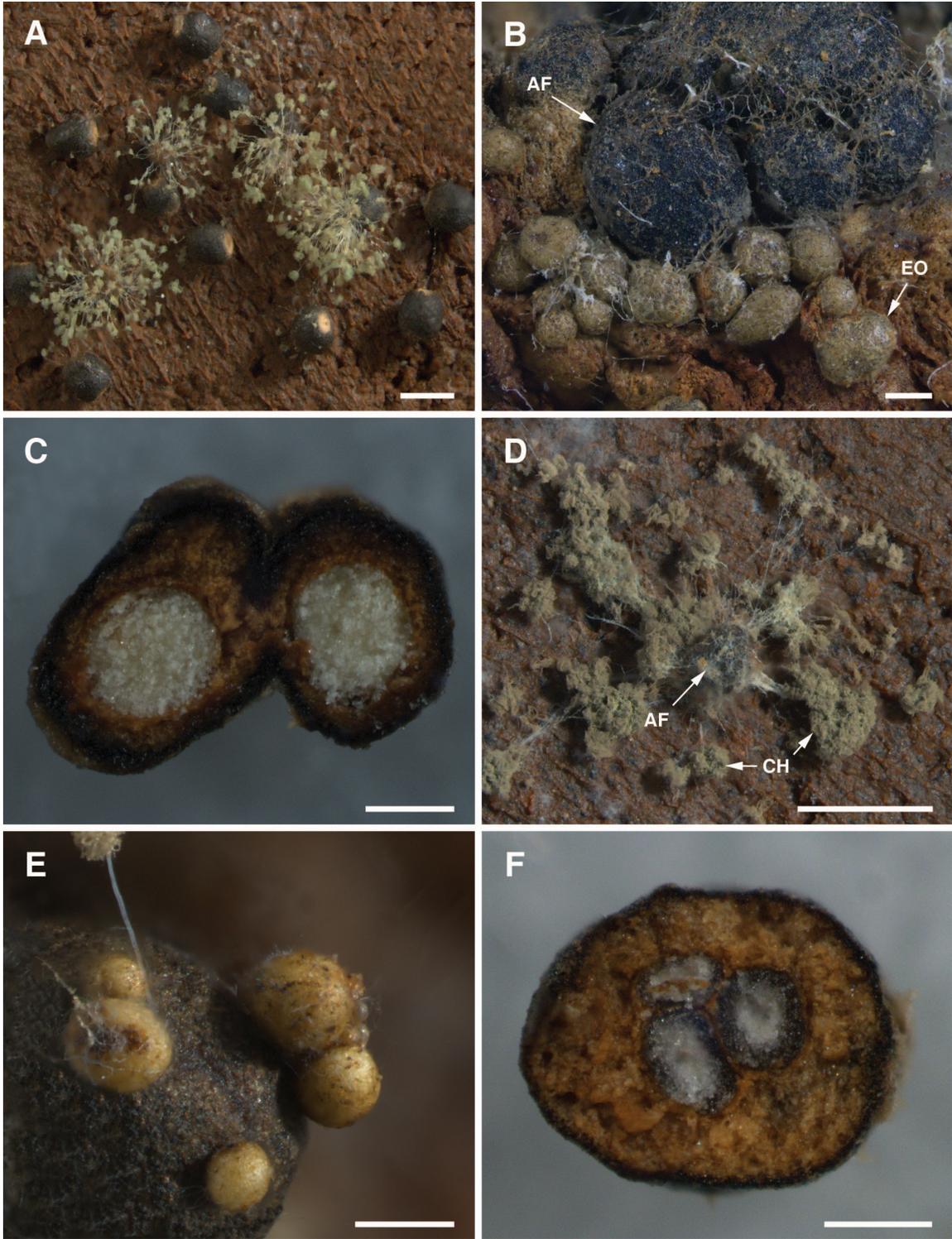
^cPercentage of sclerotia producing ascospores in parentheses.

nonsterile soil under laboratory conditions, ascospore formation was observed in 6.1% of the 6,022 sclerotia (18 of 84 ears) in 2010, 0.1% of the 2,846 sclerotia (3 of 36 ears) in 2011, and 0.5% of the 3,106 sclerotia (5 of 26 ears) in 2012 (Fig. 7.4C; Table 7.4). The majority of sclerotia that were fertile (formed ascospores) belonged to *A. flavus* L strain (Table 7.4). Fertile sclerotia of *A. flavus* S strain were detected in 2011 ($n = 1$) and 2012 ($n = 9$; distributed among 3 ears) and highly fertile sclerotia of homothallic *A. alliaceus* were detected in 2010 ($n = 114$; distributed among 2 ears). In individual ears containing exclusively *A. flavus* L strain sclerotia, fertility ranged from 0.3% ($n = 318$) to 34.6% ($n = 26$) (Table 7.4). Fertile sclerotia of *A. flavus* L strain were obtained from ears from all irrigation treatments in 2010 as well as from three of the four additional fields (Tables 7.4 and 7.5); incidences of ears with fertile sclerotia from irrigation treatments in 2011 and 2012 were very low. Ascospore germination and subsequent colony growth occurred in all ascocarps of *A. flavus* L and S strains and *A. alliaceus* from the Shellman experimental plots and the additional fields A, C and D.

Eupenicillium ochrosalmoneum sclerotia in corn.

During dissection of corn ears, *E. ochrosalmoneum* was often observed sporulating on corn kernels as well as on the conidial heads of section *Flavi*. *Eupenicillium ochrosalmoneum* produced small sclerotia inside insect-damaged kernels where they occasionally co-occurred with sclerotia of *A. flavus* (Fig. 7.4B). Ascospores were not observed in *E. ochrosalmoneum* sclerotia ($n = 772$; distributed among 16 ears) from corn ears at harvest, but 48.8% of the sclerotia ($n = 467$; distributed among 14 ears) formed ascospores after incubation on the

Figure 7.4. Reproductive structures associated with sclerotia of *Aspergillus flavus* and *Eupenicillium ochrosalmoneum*. A. Sclerotia of *A. flavus* showing sporogenic germination; sclerotia were obtained from culture (NRRL 29473 × AF36) and incubated on nonsterile soil. B. Sclerotia of *A. flavus* (AF) and *E. ochrosalmoneum* (EO) co-occurring inside an insect-damaged corn kernel. C. Sectioned fused pair of *A. flavus* sclerotia containing ascospore-filled ascocarps; sclerotia were naturally produced in corn and incubated on nonsterile soil. D. Sporogenic germination of an *A. flavus* sclerotium (AF) in which the conidial heads (CH) on the collapsed conidiophores have been colonized by *E. ochrosalmoneum*; sclerotium was obtained from corn and incubated on nonsterile soil. E. Sclerotium of *A. flavus* in which smaller *E. ochrosalmoneum* sclerotia have formed externally; *A. flavus* sclerotium was obtained from corn and incubated on nonsterile soil. F. Sectioned *A. flavus* sclerotium containing three ascospore-bearing sclerotia of *E. ochrosalmoneum*; *A. flavus* sclerotium was obtained from corn and incubated on nonsterile soil. Scale bars = 1000 µm for A and D; 200 µm for B, C, E, F.



surface of nonsterile soil. In addition, incubation of *A. flavus* sclerotia on soil often resulted in sporulation by *E. ochrosalmoneum* on the sclerotium surface or in instances of sporogenic germination, on the conidial heads produced by *A. flavus* (Fig. 4D). Ascospore-bearing sclerotia of *E. ochrosalmoneum* also were observed on the surface of *A. flavus* sclerotia (Fig. 7.4E) as well as within the matrix of *A. flavus* sclerotia where they resembled the ascocarps of *A. flavus* (Fig. 7.4F).

Discussion

Sclerotia from *A. flavus* L and S strains, *A. alliaceus* and *E. ochrosalmoneum* from corn grown under different levels of drought stress showed no evidence of sexual reproduction at corn harvest. However, subsequent incubation of sclerotia on the surface of nonsterile soil under laboratory conditions conducive to sexual reproduction, as determined by experiments using sclerotia produced from laboratory crosses, resulted in the formation of ascospore-bearing ascocarps in some of the sclerotia. This suggests that sclerotia may require an additional incubation period on soil for sexual reproduction following dispersal from corn at harvest.

Sclerotia from laboratory crosses.

Aspergillus flavus sclerotia produced from laboratory crosses generally showed similar rates of ascocarp and ascospore formation when left in culture and when detached from the parent mycelium before ascocarp formation and placed on the surface of nonsterile soil. In both instances, ascocarps were first observed after four weeks incubation and free mature

ascospores were present in ascocarps after eight weeks, which is considerably shorter than previous incubation times of 6 to 11 months (18). Therefore, the procedure for incubating sclerotia on nonsterile soil at 30°C and 100% relative humidity was appropriate for testing the ability of naturally formed sclerotia in corn to develop the sexual stage.

Sclerotia of *A. flavus* are produced by single strains as well as by crossed pairs of sexually compatible strains (17,18,33) and sclerotia from both sources are capable of germinating sporogenically. In this study, sporogenic germination often occurred in sclerotia incubated on the soil surface and depended upon the cross from which the sclerotia were derived. Wicklow and Donahue (50) also found variation in sporogenic germination in *A. flavus* and *A. parasiticus*, with sclerotia of some strains readily germinating and sclerotia of other strains not germinating or producing aerial mycelium only. Considerable variation in sporogenic germination also was observed when sclerotia from a single *A. flavus* strain were placed on the soil surface for three months in two Georgia cornfields (54). Microclimatic differences at the soil surface within a field could have been responsible for some of this variation. In this study, sporogenic germination in *A. flavus* sclerotia from crosses was associated with greatly reduced incidences of ascospore formation. Although the mechanism of mating with respect to sclerotium development is not known, it is possible that unfertilized sclerotia have a greater capacity for sporogenic germination, which is suggested by the presence of sporogenic and nonsporogenic sclerotia in close proximity on soil under the controlled conditions of this study. Alternatively, sclerotium reserves necessary to support sporogenic germination may preclude the development of a sexual stage in fertilized sclerotia.

Burial of *A. flavus* sclerotia significantly inhibited ascocarp formation, although inhibition was greater in experiment 1 compared to experiment 2. Horn and Wicklow (23) similarly showed that sclerotia of *E. ochrosalmoneum* readily produced ascospores on the surface of nonsterile soil and that ascospore formation was completely inhibited when sclerotia were buried. Viability of buried *A. flavus* sclerotia paralleled the differences between experiments in ascocarp formation, with sclerotia in experiment 1 showing both low viability and low incidence of ascocarp formation relative to experiment 2; the factors responsible for differences between experiments are not understood. When sclerotia of four strains each of *A. flavus* and *A. parasiticus* were buried for 36 months in Illinois and Georgia fields, 68 to 100% of sclerotia appeared viable when plated on agar medium yet failed to germinate sporogenically when incubated on the surface of moist sand (55). In contrast, Wicklow (49) showed that under laboratory conditions, sclerotia buried in soil saturated with water to field capacity had a viability of only 2% after 26 weeks incubation. Saturated soil with low oxygen availability reduces sclerotium viability in fungi (56) and may also inhibit the formation of the sexual stage in *A. flavus*.

Sclerotia from corn.

Drought and high temperatures are associated with increased *A. flavus* infection and aflatoxin production in corn (26,35,36) and corn kernels are most susceptible to invasion by *A. flavus* and aflatoxin accumulation during late stages of maturity (36,47). Those late developmental stages in corn occurred during July at the experimental plots in Shellman. Decreasing water availability in the irrigation treatments resulted in a significant decrease in yield for all three

years and an increase in aflatoxin concentration in 2010 and 2012; however, water availability showed no significant effect on the incidence of section *Flavi* sporulation or *A. flavus* L strain sclerotia. Aflatoxin production in corn is extremely variable due in part to its sporadic occurrence among ears and among kernels within an ear and it is estimated that in a corn lot containing 20 ppb aflatoxin B₁, only 6 of 10,000 kernels are contaminated (1,25). Fungal structures associated with aflatoxins, such as conidial heads and sclerotia, likely exhibit a similar sporadic distribution pattern among corn ears and kernels. Therefore, more extensive sampling of corn might be necessary to detect effects of corn drought stress on formation of sclerotia.

The infrequent formation of section *Flavi* sclerotia in corn (0.6% of ears) supports an earlier study in Georgia by Wicklow et al. (52) who found low densities of *A. flavus* sclerotia in corn kernels at harvest and in chaff and debris exhausted from the combine harvester. The majority of section *Flavi* sclerotia from corn in the present study comprised *A. flavus*, which is consistent with observations that *A. flavus* is the dominant aflatoxigenic species in corn (11,13). Sclerotia from *A. flavus* L strain were dominant in 2010 and 2011 from the experimental plots whereas sclerotia of *A. flavus* S strain were dominant in 2012. Aflatoxin contamination of cottonseed in southern Texas and the desert southwestern United States is largely attributed to *A. flavus* S strain, which suggests an adaptation of the fungus to high temperatures and drought (5,24). Although water availability in this study did not have a significant effect on the incidence of L strain sclerotia, an examination of S strain sclerotia over the 3-year period showed a strong association with water availability, with 81.5% of corn ears containing S strain sclerotia occurring in the 0 and 33% irrigation treatments and no

S strain sclerotia being detected in any of the 100% irrigation plots. *Aspergillus parasiticus* infrequently infects corn (11) and few ears contained *A. parasiticus* sclerotia, which have been previously reported only from peanuts (15). *Aspergillus alliaceus* is commonly isolated from soil (27) and has been reported from onions, figs, wheat, peanuts and pistachio nuts (7,8,32,40,41), but the species has not been reported in corn and this paper also represents the first report of sclerotia from nature.

Ascospore formation in sclerotia from corn.

The formation of ascocarps with viable ascospores in *A. flavus* sclerotia that were collected from corn and incubated on nonsterile soil in the laboratory demonstrates the potential for sexual reproduction in the field. Ascospore formation in sclerotia was extremely variable among corn ears as well as among sclerotia from individual ears. *Aspergillus flavus* and *A. parasiticus* might require co-infection of a crop with sexually compatible strains of the opposite mating type, an event whose low probability could account for the infrequent occurrence of ascospore formation in sclerotia. Sweany et al. (46) found a relatively low diversity of vegetative compatibility groups of *A. flavus* in corn compared to soil isolates and the majority of isolates from corn were *MATI-2*. A low infection rate by *A. parasiticus* in corn might even further reduce the ability of this species to produce fertile sclerotia.

Ascospore formation in *A. flavus* sclerotia from Shellman differed considerably for each of the three years. The 2010 and 2011 corn crops were dominated by *A. flavus* L strain sclerotia, but ascospore formation was much higher in 2010 compared to 2011. The 2012 crop was dominated by *A. flavus* S strain sclerotia and very few of those sclerotia formed

ascospores. Differences in the frequency of ascospore formation might be due to environmental conditions that influence mating during corn infection. In addition, corn is infected by soil populations of *A. flavus* that serve as a source of primary inoculum and by populations in infected crops that serve as a source of secondary inoculum (13,50). The distribution of mating types and the presence of female sterility factors (29) in either of those two populations might greatly influence the ability of *A. flavus* to mate and produce ascospores. For example, populations of *Lobaria pulmonaria* often show significant differences in the frequency of *MAT1-1* and *MAT1-2* genes and the predominance of one mating type lowers the likelihood of sexual reproduction in those populations (43). Horn et al. (21) postulated that sclerotia serve a dual purpose in the life cycle of *A. parasiticus* by (i) withstanding adverse environmental conditions (unfertilized sclerotia) and (ii) providing for genetic recombination (fertilized sclerotia). Data presented here suggest that the majority of *A. flavus* sclerotia are not involved in sexual reproduction and instead act as resistant structures or produce conidia through sporogenic germination.

Ascocarps and ascospores were not detected in *A. flavus* sclerotia at corn harvest. In addition, sclerotia retrieved from homothallic *A. alliaceus* and *E. ochrosalmoneum* at harvest also did not contain ascospores. However, *A. alliaceus* and *E. ochrosalmoneum* sclerotia both readily formed ascospores after laboratory incubation on nonsterile soil, suggesting that sclerotia of homothallic species as well as sclerotia of mated heterothallic *A. flavus* and *A. parasiticus* require an additional incubation period on soil after dispersal for development of the sexual stage. *Aspergillus flavus* sclerotia from corn in this study were incubated on soil at 30°C and 100% relative humidity, but the conditions conducive to ascospore formation in

nature, where sclerotia are exposed to daily and seasonal fluctuations in temperature and humidity, are not known. Ascospore formation in *E. ochrosalmoneum* sclerotia on soil is highly temperature dependent, with an optimum rate of formation at 25 to 30°C; ascospores are not produced at 15 and 37°C (23).

The present study showed that asexual sporulation by *E. ochrosalmoneum* on the conidial heads of *A. flavus* occurs in corn under field conditions. In culture *E. ochrosalmoneum* demonstrates high host specificity for section *Flavi* in its ability to sporulate on the conidial heads of *Aspergillus* (20). Horn (12) speculated that *E. ochrosalmoneum* uses the much taller conidiophores of *Aspergillus* as a platform for aerial dispersal of conidia. When *A. flavus* sclerotia from corn were incubated on soil, *E. ochrosalmoneum* not only sporulated asexually on the surface of sclerotia and on the conidial heads arising from sporogenic germination, but also formed ascospore-bearing sclerotia on the surface or within the matrix of *A. flavus* sclerotia. It is not known whether *A. flavus* sclerotia were colonized by *E. ochrosalmoneum* during development on corn or after placement on soil. Internally produced sclerotia of *E. ochrosalmoneum* could be distinguished from ascocarps of *A. flavus* by the thicker outer wall and by ascospores that are $3.5\text{-}5.0 \times 2.5\text{-}3.5 \mu\text{m}$ with two longitudinal flanges (38), as opposed to the ascospores of *A. flavus* which are $8.0\text{-}12.5 \times 7.5\text{-}12.0 \mu\text{m}$ and have a single equatorial ridge (18). Although the nature of the relationship between *E. ochrosalmoneum* and section *Flavi* has not been elucidated (12,20), the formation of *E. ochrosalmoneum* sclerotia within the matrix of *A. flavus* sclerotia suggests that parasitism is involved. By forming sexual structures within the

larger *A. flavus* sclerotia, *E. ochrosalmoneum* benefits from any protective and dispersal properties provided by the host sclerotium.

Future research.

Much remains to be learned about the sexual cycle of heterothallic *A. flavus* and the contribution of sexuality to genetic diversity in agricultural fields. In this study, a small proportion of *A. flavus* strains of the opposite mating type likely became associated during infection of corn. However, the development of sclerotia in relation to mating and nuclear fusion is not understood and additional research is needed to determine whether these initial stages of the sexual process occur in the crop or after dispersal onto soil. Furthermore, the role of soil microbial populations during sexual development and the environmental conditions most conducive to sexual reproduction in nature have not been examined. Natural populations of *A. flavus* show an evolutionary history of recombination based on patterns of linkage disequilibrium (LD) in the aflatoxin gene cluster (31). In a single generation, progeny from laboratory crosses exhibit crossing over in the aflatoxin gene cluster that results in LD blocks consistent with patterns of LD in natural populations (33). The frequency and patterns of recombination in progeny from naturally produced ascospore-bearing sclerotia remain to be determined.

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CHAPTER 8

MITOCHONDRIAL GENOME SEQUENCES REVEAL EVOLUTIONARY RELATIONSHIPS OF THE *PHYTOPHTHORA IC CLADE SPECIES*

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Key Words

Phytophthora I c clade, mitochondria, Late blight, phylogenetics

Abstract

Phytophthora infestans is one of the most destructive plant pathogens of potato and tomato globally. The pathogen shares a clade with four other species including *P. phaseoli*, *P. ipomoeae*, *P. mirabilis*, and *P. andina*. We have sequenced mitochondrial genomes of the sister species of *P. infestans* in order to resolve the evolutionary relationships within the clade. We observed that both gene order and content are conserved among species with the exception of a 12 nucleotide deletion in coding genes in ORF79 of *P. andina*. Bayesian phylogenetic analysis indicates that the *P. phaseoli* mitochondrial lineage is basal within the clade. *P. mirabilis* and *P. ipomoeae* are sister lineages and share a common ancestor with the Ic mitochondrial lineage of *P. andina*. These lineages in turn are sister to the *P. infestans* and *P. andina* Ia mitochondrial lineages. More recent divergences led to the *P. infestans* and *P. andina* Ia mitochondrial lineages. Our data are consistent with a South American center of evolutionary origin for all the species in the clade since *P. andina*, *P. infestans* and *P. phaseoli* coexist in the Andean region of Ecuador. We cannot rule out a Mexican origin, however, since *P. andina* has not been found in Mexico, this scenario is unlikely. Further surveys are needed to test this hypothesis and the possibility that *P. mirabilis* and *P. ipomoeae* exist in the Andean region.

Introduction

Phytophthora infestans (Montagne) de Bary, the destructive pathogen responsible for the Irish potato famine of the mid-1800's (Bourke, 1964) still causes widespread disease on both potato and tomato (Hu *et al.*, 2011; Fry *et al.*, 2013). Although originally placed in the Kingdom Fungi, *P. infestans* and other oomycetes were shown to be more closely related to members of the Kingdom Stramenopila than the Kingdom Fungi following analysis of the mitochondrial *nad4L* subunit of the NADH dehydrogenase complex (Chesnick *et al.*, 1996) and the nuclear small subunit rRNA (Gunderson *et al.*, 1987; Föster *et al.*, 1990).

Phytophthora infestans is grouped in Waterhouse's morphological group IV, which includes both heterothallic (outcrossing) and homothallic (inbreeding) species (Waterhouse 1963).

Cooke *et al.*, (2002) conducted a phylogenetic analysis of 50 *Phytophthora* species based on the internal transcribed spacer regions of the rRNA genes and resolved 8 clades. Subsequent studies with additional species utilized both nuclear and mitochondrial genes and current phylogenies divide the genus *Phytophthora* into ten main clades and supported the phylogeny reported by Cooke (Kroon *et al.*, 2004; Blair *et al.*, 2008).

Phytophthora infestans reproduces sexually in central Mexico which is a center of diversity of the pathogen. *P. infestans* shares clade Ic with four other closely related species including *P. phaseoli*, *P. ipomoeae*, *P. mirabilis*, and *P. andina*. *P. phaseoli*, is a species with a wide geographic range in the Andean and MesoAmerican region and infects *Phaseolus lunatus*. *P. mirabilis*, causes disease on *Mirabilis jalapa* (Galindo and Hohl, 1985) and *P. ipomoeae* causes leaf blight on *Ipomoea longipedunculata* and are both species have been reported only in Mexico (Flier *et al.*, 2002). The Toluca Valley in central Mexico

has been proposed as the center of origin of the entire Ic clade (Brasier and Hansen 1992; Flier *et al.*, 2002; Flier *et al.*, 2003; Goodwin *et al.* 1992; Grunwald and Flier 2005; Niederhauser 1991). *P. infestans*, *P. mirabilis* and *P. ipomoeae* share morphological characteristics with the exception of heterothallism in *P. infestans* and *P. mirabilis* and homothallism in *P. ipomoeae*, and lack ITS diversity. It has been suggested that sympatric evolution occurred in the highlands of central Mexico. Host jumps followed by host specialization through mutations of effector genes may be driving speciation in the clade (Flier *et al.*, 2002; Raffaele S, *et al.*, 2010).

In Ecuador, three clonal lineages of *P. infestans* (US-1, EC-1, EC-3) and one heterogeneous lineage consisting of two mitochondrial haplotypes (EC-2) named *Phytophthora infestans sensu lato* are found in association with different wild *Solanum* species (Adler *et al.*, 2004; Ordonez *et al.*, 2000). Kroon *et al.*, (2004) sequenced several nuclear and mitochondrial genes from the EC 2 Ic mitochondrial lineage and placed this lineage in the *Phytophthora* Ic clade and first used the name “*P. andina*”. Subsequently, Blair *et al.*, (2008) referred to the species as “*P. sp. ‘andina’*”, however, the evolutionary relationships between the five species remained unresolved (Kroon *et al.*, 2004).

The EC-1 clonal lineage of *P. infestans sensu lato* was later determined to be *P. infestans* based on sequences of the mitochondrial cytochrome oxidase I (*cox I*) gene and intron 1 of the *ras* gene (Gómez-Alpizar *et al.*, 2008). However, the EC-2 1c lineage formed a branch in the Ic clade distinct from *P. infestans*, *P. mirabilis*, *P. phaseoli* and *P. ipomoeae*, for both *cox I* and *ras* intron 1, and these isolates were identified as the newly described species *P. andina* (Gómez-Alpizar *et al.*, 2008). Oliva *et al.*, (2010) published a formal

species description of *P. andina* and suggested that in addition to the EC-2 1c lineage, that the EC-3 lineage was also *P. andina*. Some authors have questioned the validity of *P. andina* as a species (Cardenas *et al.*, 2012), while others have suggested a hybrid origin of the species (Forbes *et al.*, 2012; Gomez *et al.*, 2008; Goss *et al.*, 2011). Limited molecular evidence from several nuclear and mitochondrial genes suggests that *P. andina* could have arisen via hybridization between *P. infestans* and either *P. mirabilis* (Gomez *et al.*, 2008) or *P. infestans* and a close relative of *P. mirabilis* that is a yet unknown species (Goss *et al.*, 2011). Blair *et al.*, (2012) recently resolved another multilocus species tree of the clade and suggested that *P. andina* is a hybrid and more closely related to *P. ipomoeae*.

P. mirabilis is closely related to *P. infestans* and was first described on *Mirabilis jalapa* also known as 4'o clock or the "Flower of Peru" in Mexico (Galindo and Hohl 1985; Goodwin and Fry 1994). Studies have shown that *P. infestans* and *P. mirabilis* are now reproductively isolated but the possibility of gene flow between the species was demonstrated since the two species can interbreed and produce viable offspring (Gómez-Alpizar *et al.*, 2008; Goodwin *et al.*, 1999). *P. andina*, however, is native to the Andean region of South America in Ecuador, and presently has not been found in Mexico where *P. infestans* and *P. mirabilis* are sympatric. Thus, the hybridization would have had to occur in the Andes, suggesting a South America origin of *P. andina* as has been proposed for *P. infestans* (Gómez-Alpizar *et al.*, 2007). The oldest mutations in the lineage leading to *P. andina* are of South American origin (Gomez *et al.*, 2007). Conversely, *P. mirabilis* does not appear to be conspecific with *P. infestans* anywhere except Mexico. *Mirabilis jalapa* occurs widely in South America, but has not been surveyed for infection by *P. mirabilis*. Several

plants including pear melon (*Solanum muricatum*) and tree tomato (*S. betaceum*) are also hosts to both *P. infestans* and *P. andina* (Adler, 2004)

In this study we have further investigated the evolutionary history of *Phytophthora* species in the Ic clade by: 1) sequencing and annotating the complete mitochondrial genomes of the remaining four species of the *Phytophthora* Ic clade: *P. mirabilis*, *P. ipomoeae*, *P. phaseoli*, and both mitochondrial lineages of *P. andina* (Ia and Ic); 2) comparing the genomes to the mitochondrial genomes of *P. infestans*; and 3) conducting statistical analysis to resolve the evolutionary relationships among species in the clade. A preliminary abstract of a portion of this research has been published (Lassiter *et al.*, 2010).

Materials and Methods

Isolates

Five isolates of *Phytophthora* were used for this study including: *P. mirabilis*, *P. ipomoeae*, *P. phaseoli*, and *P. andina* (mitochondrial lineages Ic and Ia) (Table 8.1). The mitochondrial genome sequence of the *P. infestans* Ib mt haplotype (NC 002387) was used as the reference genome for comparison (Lang end Forget, 1993). All isolates were maintained on rye-V8 or lima bean agar at 18°C. Two to three agar plugs containing mycelia were transferred into Petri dishes containing pea broth with 0.5 g/L sucrose and grown for 2-3 weeks. Mycelia from approximately 40 Petri dishes was harvested by filtration with Whatman #1 membrane filter paper, and then frozen in liquid nitrogen. Mycelia were stored at -20°C until further use. Approximately 10g of mycelia was used for DNA extraction.

Table 8.1. Isolates and GenBank accession numbers of *Phytophthora species* used for mitochondrial genome sequencing.

Species ^a	Isolate	Collection ^b	Origin	GenBank accession number
			West	
<i>P. infestans</i>	West Virginia 4	ATCC16981	Virginia	NC002387
<i>P. phaseoli</i>	Phy P18	P11078	Delaware	HM590418
<i>P. andina</i> (Ic)	EC 3425	CBS ^b 122202 ^c	Ecuador	HM590419
<i>P. andina</i> (Ia)	EC 3394		Ecuador	KJ408269
<i>P. ipomoeae</i>	Pic 99167	CBS 122203	Mexico	HM590420
<i>P. mirabilis</i>	Pic 99114	CBS 122204	Mexico	HM590421

^a *P. infestans* (Ib), US-1 lineage, Lang and Forget, 1993; *P. andina* (Ic) EC-2 lineage, Adler et al, 2004; *P. andina* (Ia) EC-3 lineage, Adler et al, 2004; *P. ipomoeae*, Flier et al, 2002; and *P. mirabilis*, Galindo and Hohl, 1985.

^b ATCC- American Type Culture Collection, Manassas, Virginia; CBS- Fungal Diversity Centre, Netherlands; ^c World *Phytophthora* and Oomycete Genetic Resource Collection, UC Riverside.

DNA Preparation

Previously collected mycelia were frozen in liquid nitrogen and then ground into a fine powder using a mortar and pestle. DNA extraction was performed using the Qiagen DNeasy Plant Maxi® kit (Qiagen Corporation, Maryland, USA). The mtDNA was purified in a cesium chloride (CsCl) density gradient (1.1g CsCl/mL DNA-AE solution) with the addition of bisbenzimidazole (final concentration of 10 mg/mL) as described previously (Avila-Adame *et al.*, 2006; Carter *et al.*, 1990; Garber and Yoder 1983; Klimczak and Prell 1984). The DNA was purified by two consecutive centrifugations in a Beckman ultracentrifuge with a 70.1 Ti rotor at 55K for 42-48 hours at 20°C. The bisbenzimidazole and CsCl were removed with the use of isopropanol and dialysis in TE buffer (at 4°C for 16 hours including three changes of buffer), respectively. The mtDNA was concentrated by ethanol precipitation in the presence of 1/10 3.0 M sodium acetate (pH 5.2), and resuspended by dissolving in AE buffer provided in the Qiagen kit. The *P. andina* Ia mitogenome was prepped using a standard CTAB extraction (Ristaino *et al.*, 2001).

Sequencing

The mitochondrial genomes of *P. ipomoeae*, *P. phaseoli* and *P. andina* (1c haplotype) were sequenced at the BROAD Institute of MIT with a minimum of 100-fold sequence coverage with standard 454 technology fragment reads using methods described previously (Lennon *et al.*, 2010). The mtDNA was sheared and size selected to generate fragments from 400 to 800 bp in length. Fragments were blunt ended, ligated on both ends with 454 adapter sequences, and the resulting library was subjected to emulsion PCR. The DNA was then sequenced

using the 454 Titanium sequencing platform. The *P. andina* Ia genome was sequenced at the Centre for GeoGenetics, University of Copenhagen. Initially DNA was fragmented using a Bioruptor to a mean size of 200bp, following which an Illumina compatible library was constructed using the NEBNext library build kit 1 and Illumina PE adaptors, following the manufacturer's instructions. The library was subsequently index PCR amplified (Illumina index 1) for 20 cycles using Pfusion polymerase, prior to gel excision, quantification, and sequencing over 1 lane of Illumina GAIIx (SR 76bp) and 1 lane of Illumina HiSeq (SR100bp) following the manufacturer's guidelines.

Annotation

The 454 reads were reassembled using Newbler (Roche). The identification of genes, open reading frames, and other features in the mitochondrial genomes was based on the previously published mitochondrial genome sequence of *P. infestans* (mtDNA haplotype Ib available in GenBank accession no. NC002387). Portions of the four genomes were aligned with the *P. infestans* genome using BioEdit 7.0.5.3 (Hall 1999). Each gene within the *P. infestans* genome was locally aligned with the entire genome of each of the remaining species. The mitochondrial genomes of each species were analyzed and mapped using the software program Vector NTI suite 9.0. Annotated sequence data was submitted to GenBank and accession numbers are reported (Table 8.1).

The *P. andina* Ia genome was annotated using the *P. andina* Ic mitochondrial genome. AdapterRemoval (Lindgreen 2012) was used to trim adaptors, N bases and low quality sequence from the raw Illumina reads using default parameters. Sequences were

initially mapped using BWA against the *P. andina* Ic mitochondrial genome (HM590419). Subsequently, duplicate reads were removed using samtools (Li *et al.*, 2009) and reads that mapped to more than one position were removed using shell scripts. The GATK pipeline (McKenna *et al.*, 2010) was then used to realign the reads and to call SNPs and indels, following which a consensus sequence was generated using custom perl scripts. Bases were called consensus if a called SNP was present in more than 70% of the reads mapping to one position. Post trimming, 46,184 correctly indexed reads mapped uniquely to the reference, resulting in an assembly with 91.08x mean coverage (median 71x), and covering 99.735% of the reference at minimum 1x.

Evolutionary Analysis

Mitochondrial genome sequences were aligned using Vector NTI, edited, trimmed and collapsed into haplotypes using SNAP Map (Aylor *et al.* 2006) with the options of removing insertions and deletions and excluding infinite-sites violations. Base substitutions were categorized as transitions/transversions and phylogenetically informative/uninformative. The presence of all four possible haplotypes for a pair of diallelic sites results in incompatibility, which is an indication of either homoplasy or recombination (Hudson and Kaplan 1985). Compatibility among pairs of informative sites and the largest nonrecombining partition were determined using the Clade and CladeEx programs (Bowden *et al.* 2008), respectively. Mitochondrial ancestral history was based on sequence variation in the largest nonrecombining partition and gene genealogies inferred from haplotype coalescence using

Genetree version 9.0 (Griffiths and Tavaré 1994) as implemented in SNAP Workbench (Price and Carbone 2005; Carbone *et al.*, 2004).

Relationships among the species were also determined by using Bayesian inference to construct gene trees in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) run for 1000000 generations of Markov Chain monte Carlo with a burn-in of 1000, gamma distribution, and infinite sites substitution model (Supplemental Figure 8.1). Although the Ic clade does not match all assumptions of these coalescent-based techniques (*e.g.*, the assumption of a single randomly mating population with no subdivision), we do not expect the violations of assumptions to cause incorrect rooting of the haplotype tree or the incorrect mapping of substitutions to branches on the tree.

Analysis was also conducted using BEAST (Drummond and Rambaut 2007) to confirm the findings in SNAP workbench. An xml file was created in BEAUti with simulations run for 1000000 generations of Markov chain Monte Carlo with a burn in of 1000 under an infinite sites model with gamma distribution. Trees were summarized into a consensus tree using TreeAnnotator, that tree was visualized in TreeView 1.6.6 (Page 1996).

Results

We completed the mitochondrial genome sequencing of *P. andina*, *P. mirabilis*, *P. ipomoeae*, and *P. phaseoli*. The genome sizes are presented in Table 8.2. The mitochondrial genome sequence for the Ib haplotype of *P. infestans* was used for comparison (Lang and Forget 1993; Paquin *et al.*, 1997). The mitochondrial genomes of the *P. andina* lineages, *P.*

Table 8.2. Mitochondrial genome size and content of *Phytophthora* species in Ic clade.

Species	Genome size (bp)	% Identity ^a	Coding		Non-coding		G + C Content (%)
			bp	%	bp	%	
<i>P. infestans</i> (Ib)	37957	-	34209	90.1	3748	9.9	22.3
<i>P. phaseoli</i>	37914	99.0	34284	90.4	3630	9.6	22.4
<i>P. andina</i> (Ia)	37883	99.9	34272	90.4	3611	9.5	22.2
<i>P. andina</i> (Ic)	37874	99.3	34272	90.4	3602	9.5	22.1
<i>P. ipomoeae</i>	37872	99.3	34284	90.5	3670	9.7	22.4
<i>P. mirabilis</i>	37778	99.4	34284	90.7	3494	9.2	22.4

^a% identity is calculated from alignment of only coding regions and compared to *P. infestans*.

ipomoeae, and *P. phaseoli* were 83, 74, 85, and 43 bp smaller than the mitochondrial genome of the *P. infestans* Ib haplotype.

The genome sizes for species in the Ic clade ranged in size from 37,778 to 37,957 bp (Table 8.2). The largest differences in mitochondrial genome sizes were observed between *P. infestans* and *P. mirabilis* (Table 8.2). *P. mirabilis* had the smallest mitochondrial genome and *P. infestans* had the largest mitochondrial genome. The mitochondrial genome of *P. mirabilis* was 179 bp smaller than that of the Ib mitogenome of *P. infestans*.

Coding sequence identity between *P. infestans* Ib mitogenome and each of the other Ic clade mitochondrial genomes was greater than 99% (Table 8.2). The *P. andina* Ia mitogenome had the greatest sequence similarity to the *P. infestans*. All five genomes were adenine and thymine rich and G-C content was less than 23% in each of the species (Table 8.2). The coding regions in all of the genomes accounted for more than 90% of the mitogenomes (Table 8.2). A total of 61 genes with known function were identified including: 18 genes involved in electron transport, 2 ribosomal RNA genes, 16 ribosomal protein genes, and 25 transfer RNA genes (Table 8.3). Six ORFs with unknown function were also identified. Genes were coded in both strands of the DNA, and gene order was identical among all five Ic clade species (Figures 1A-D, Supplemental Table 1). None of the ORFs identified in the type II mitochondrial genomes of *P. infestans* (Avila-Adame *et al.*, 2006) were found among the four sister species. The genes coding for the ribosomal proteins *rps12* and *rps7* overlapped and share 71 base pairs in all species sequenced. Similarly, there was an observed overlap between the genes coding for the NADH dehydrogenase subunits 1 (*nad1*) and 11 (*nad11*); these genes share 4 base pairs. Two indels were detected in both

Table 8.3. Gene content of the mitochondrial genomes of five species of the *Phytophthora* Ic clade^a. Genes were defined according to Paquin et al. (1997).

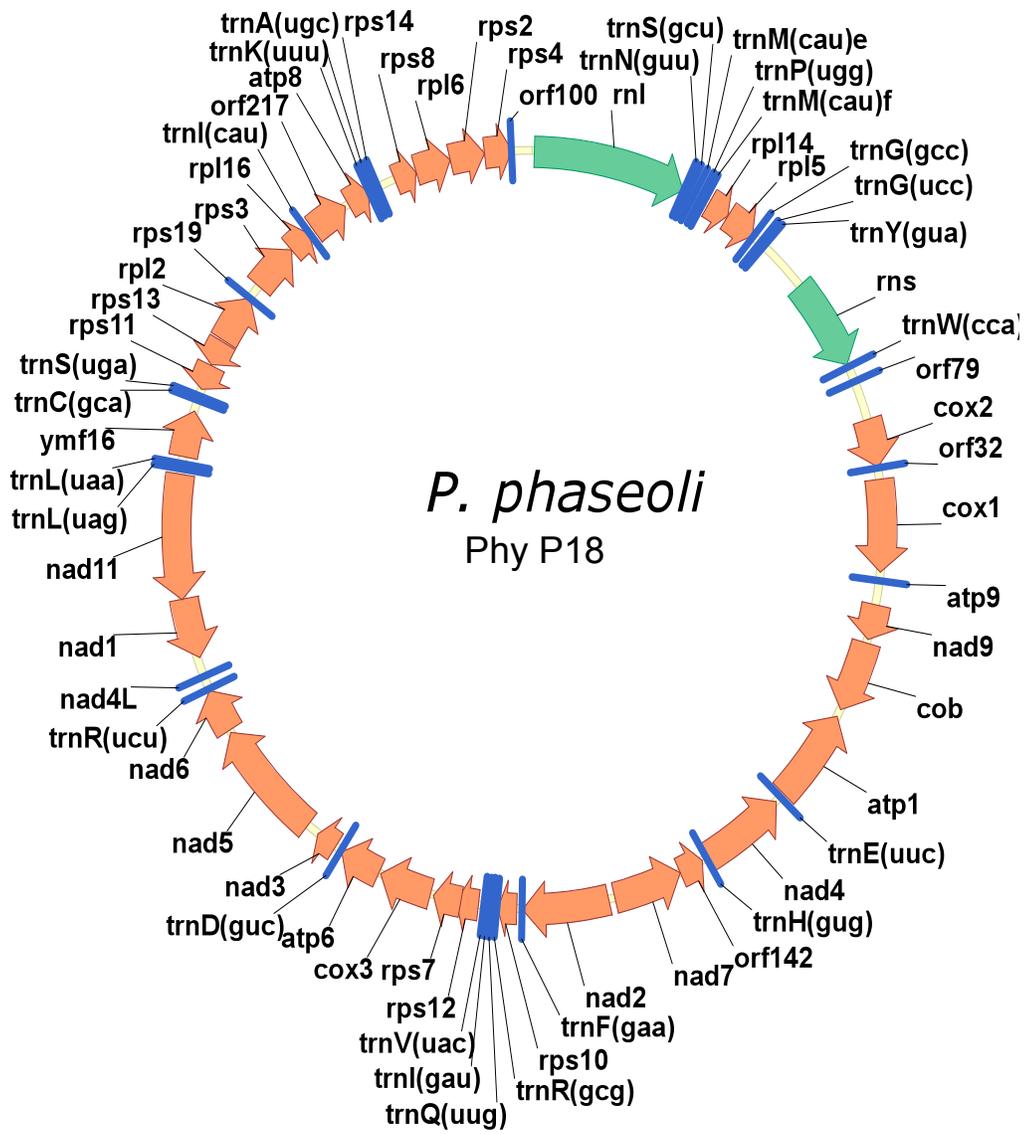
	Ic Clade Species				
	<i>P. infestans</i>	<i>P. mirabilis</i>	<i>P. andina</i>	<i>P. ipomoeae</i>	<i>P. phaseoli</i>
Genes involved in electron transport and coupled oxidative phosphorylation	18	18	18	18	18
RNA-encoding genes	2	2	2	2	2
Ribosomal protein genes	16	16	16	16	16
Conserved ORFs ^b	6	6	6	6	6
Unique ORFs	0	0	0	0	0
Transfer RNA genes	25	25	25	25	25

^a Fig. 1 shows the distribution of genes in the genome.

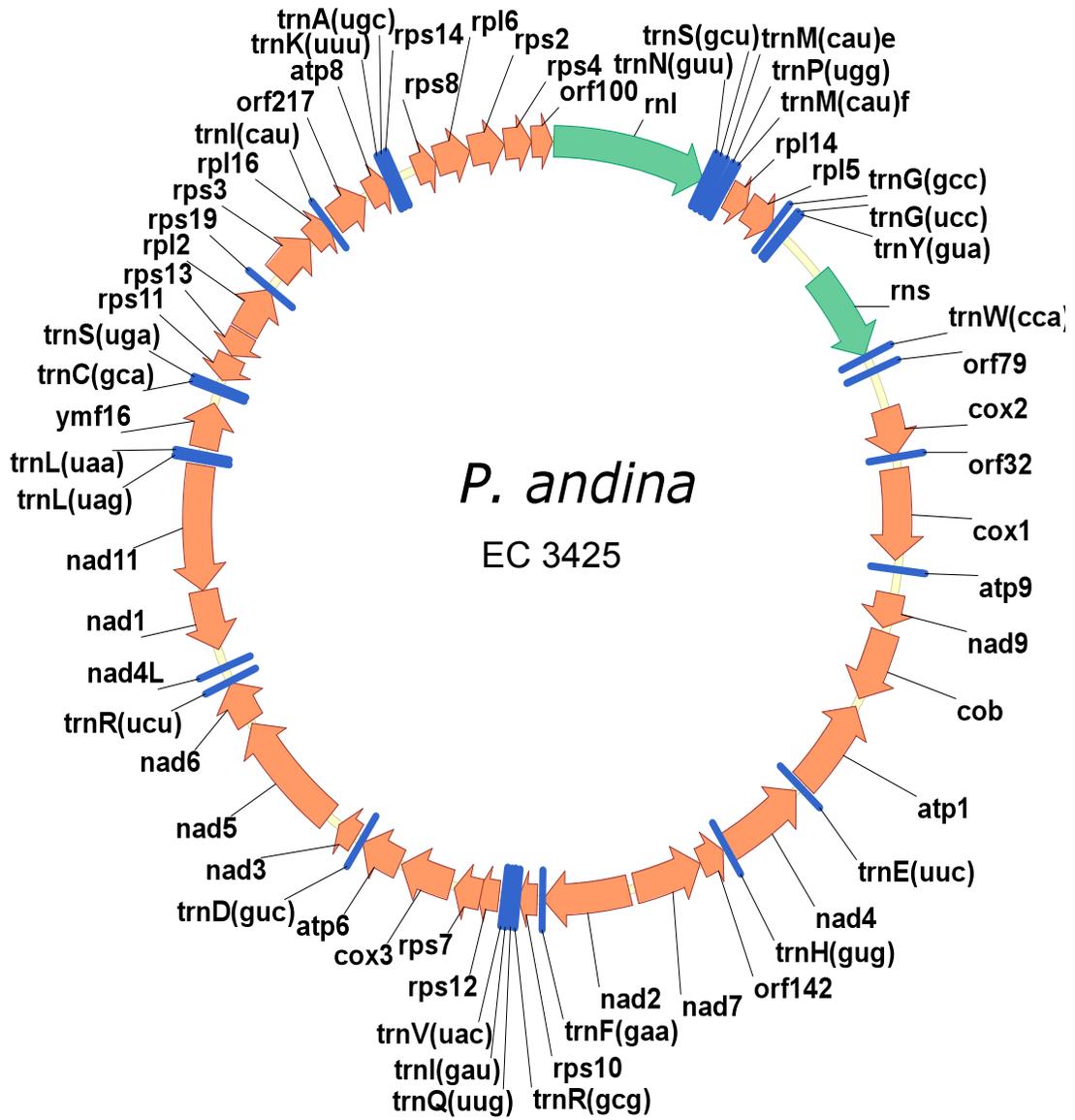
^b The conserved ORFs include ymf16, which codes for secY-independent transporter, ORFs 32, 79, 100, 142, and 217.

Figure 8.1. Mitochondrial genome maps of the *Phytophthora* Ic clade (A) *P. phaseoli* 37,914 bp; (B) *P. andina* Ic with a size of 37,874 bp, Ia is 37,883 bp; (C) *P. mirabilis* 37,778 bp; and (D) *P. ipomoeae* 37,872 bp. Gene order and size are both conserved among the species; genome size is also highly similar.

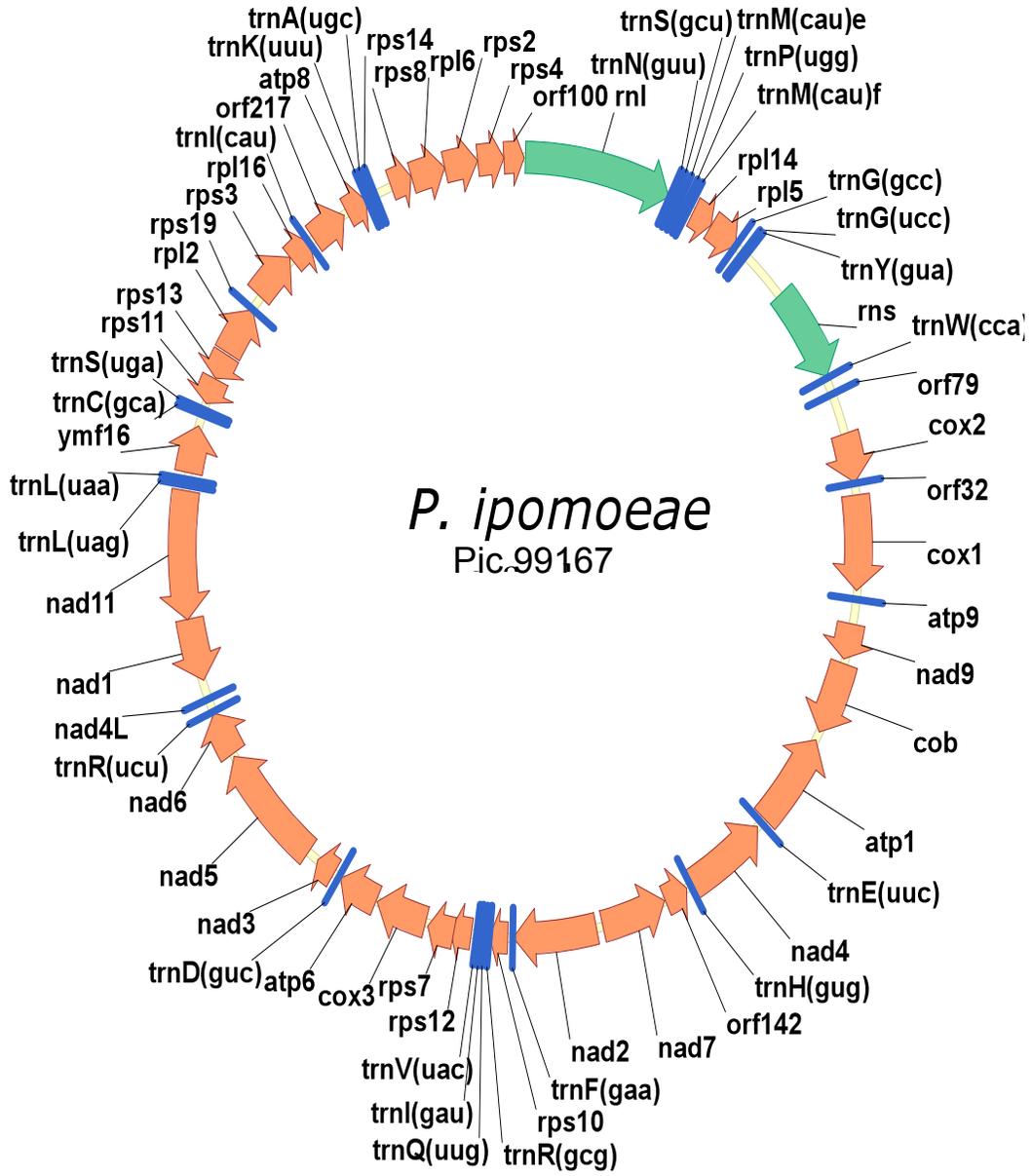
A



B



C



haplotypes of *P. andina* ORF79 corresponding to a 12 nucleotide deletion in the region (Figure 8.2). These indels were not found in any of the genomes of the other Ic clade species and do not correspond to any of the known restriction enzyme sites used to differentiate the *P. andina* Ic mitochondrial lineage from *P. infestans* (Ordonez *et al.*, 2000).

A total of 672 polymorphic sites were detected among the mitochondrial genomes of five species in the clade (Table 8.4, Supplemental Figure 8.2). Among these polymorphic sites were 198 transversions and 474 transitions (Supplemental Figure 8.2). A total of 129 replacement substitutions were identified (Supplemental Table 8.2). None of these polymorphic sites produced frameshifts; however, a 12-nucleotide (4 amino acid) deletion in *P. andina* ORF79 yielded a truncated version of the ORF (Figure 8.2).

Coalescent analysis using Genetree was used to infer the mutational history, time scale, and evolution of the polymorphism among the species. The largest nonrecombining partition was collapsed into nine haplotypes and 633 variable sites (Supplemental Figure 8.2). Only regions corresponding to coding genes, tRNAs, or rRNAs were used for coalescent inference. The relative probabilities of all rooted genealogies were calculated by performing four independent runs of 1–10 million simulations of the coalescent assuming Watterson's (1975) estimate of theta, panmixis and constant population size. The mitochondrial genealogy with the highest root probability was examined to determine the relative ages of polymorphisms and clades in the evolutionary history of the species.

The phylogenetic analysis indicates that *P. phaseoli* is the basal lineage of the clade (Figure 8.3, Supplemental Figure 8.3). The *P. infestans* and *P. andina* Ia mitochondrial lineages share a common ancestor with the lineages leading to the remaining three species

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          10      20      30      40      50      60      70      80
P. andina Ic ORF79 ATGCAAAAAAAAAATTAATAATTTTATT-----TTTATTCTTAAGTATAAGCAT-----TTTTATCTTATACTTACATAACG
P. andina Ia ORF79 ATGCAAAAAAAAAATTAATAATTTTATT-----TTTATTCTTAAGCATAAGCAT-----TCTTATCTTATACTTACATAACG
P. infestans ORF79 atgcaaaaaaaattaaaaattttattcattttttatttttaagtataagcataagcattccttattctatacttacataaacg

          110     120     130     140     150     160     170     180
P. andina Ic ORF79 TAAAAATTATATTTTTATTATTAAAAACAGAATTAATATCTTTACTCTATGTATAGATGATGACCACATTCATCCACGTTA
P. andina Ia ORF79 TAAAAATTATATTTTTATTATTAAAAACAGAATTAATATCTTTACTCTATGTATAGATGATGACCATTTTCATCCACGTTA
P. infestans ORF79 taaaaattatatttttattattaaaaaacagaattaatatctttactctatgtatagatgatgaccattttcatccacgtta

          210     220     230     240
P. andina Ic ORF79 TAATTTATTAATTATGGAATTATCAGAAGATTTTTCTTAA
P. andina Ia ORF79 TAATTTATTAATTACGGAATTATCAGAAGATTTTTCTTAA
P. infestans ORF79 taatttattaattacggaattaatcggaagatttttcttaa

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Figure 8.2. Schematic of *P. infestans* ORF79 and *P. andina* ORF 79 showing a 12 nucleotide deletion present only in *P. andina*.

Table 8.4. Number of polymorphisms^a found among the *Phytophthora* Ic clade species.

Species	<i>P. infestans</i>	<i>P. phaseoli</i>	<i>P. andina</i> (Ic)	<i>P. andina</i> (Ia)	<i>P. ipomoeae</i>	<i>P. mirabilis</i>
<i>P. infestans</i>	0	337	225	14	238	189
<i>P. phaseoli</i>	-	0	364	343	380	344
<i>P. andina</i> (Ic)	-	-	0	226	241	199
<i>P. andina</i> (Ia)	-	-	-	0	239	191
<i>P. ipomoeae</i>	-	-	-	-	0	213
<i>P. mirabilis</i>	-	-	-	-	-	0

^aPolymorphic sites include 672 single base pair substitutions within coding sites; these sites do not include indels greater than one bp.

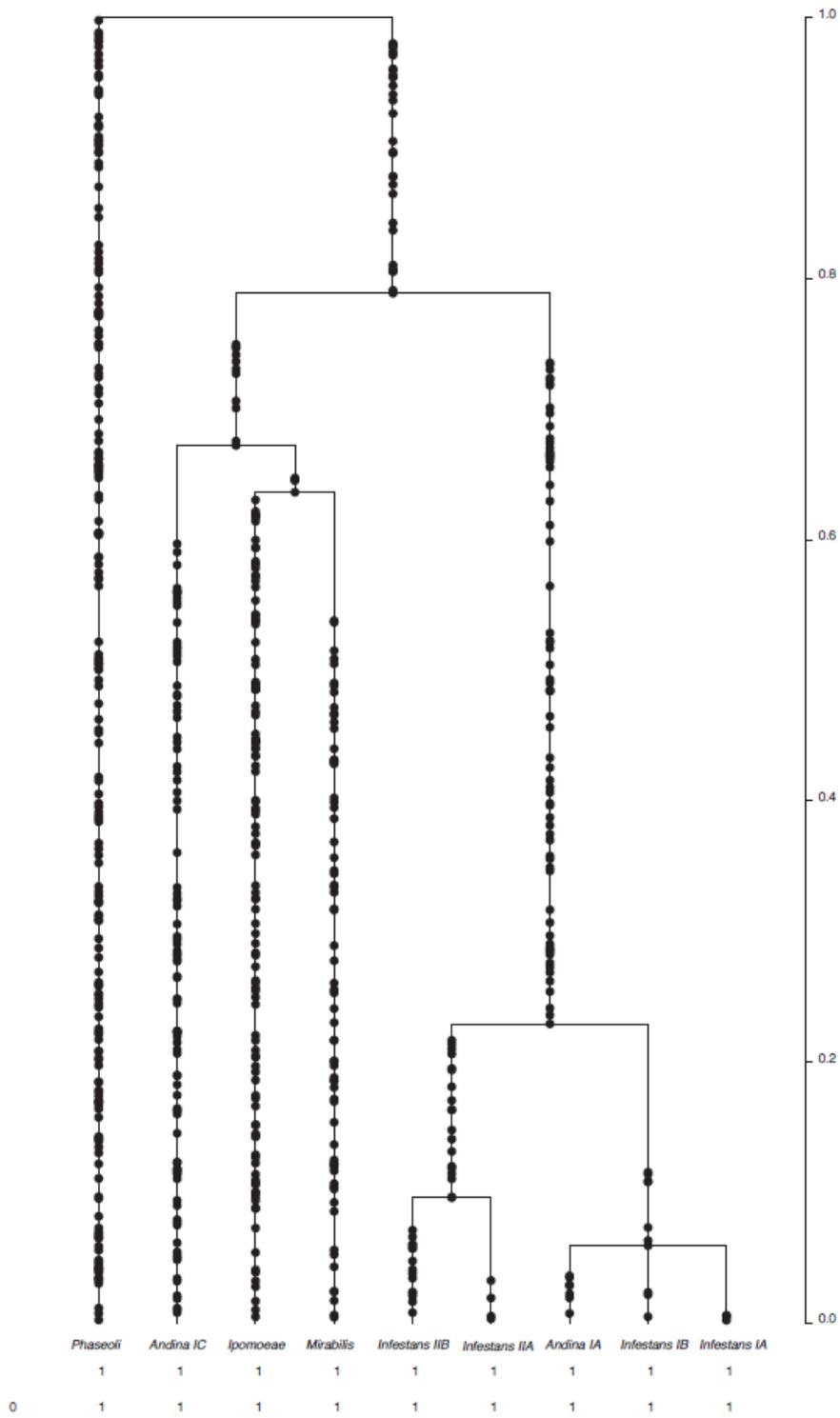
(*P. andina* Ic, *P. ipomoeae*, and *P. mirabilis*). The *P. andina* Ic lineage shares a common ancestor with *P. mirabilis* and *P. ipomoeae*. *P. mirabilis* and *P. ipomoeae* are inferred to be sister lineages distinct from the *P. andina* Ic lineage (Figure 8.3). The most recent diverged lineages in coalescent time contained the four mitogenomes of *P. infestans* and the *P. andina* Ia mitogenome,

Each branch on the haplotype tree indicated multiple fixed substitutions that accumulated in the mitochondrial genome. *P. phaseoli* had the greatest number of fixed substitutions (190) and this number was higher than any other species of the clade. *P. ipomoeae* had the second highest number of fixed substitutions (112), followed by the *P. andina* Ic lineage (94) and *P. mirabilis* (69). The lineage that led to the separation of the other five members of the clade from *P. phaseoli* included 28 substitutions (Supplemental Figure 8.3). The tree resolved using BEAST has the identical topology to the coalescent tree (Supplemental Figure 8.3)

Discussion

Analysis of mitochondrial genomes of *Phytophthora* species, including *P. ramorum* and *P. sojae*, has shown that there is a high degree of gene conservation in the genus (Martin *et al.*, 2007). This degree of gene conservation was also observed among the *Phytophthora* species in the Ic clade. Both gene content and order were identical amongst the five species in the clade. Since the mitochondrial genome is a small organelle, there are many important conserved coding genes contained within it (Martin *et al.*, 2007). No unique ORFs or insertions were found amongst the Ic clade species of *Phytophthora*, with the exception of *P.*

Figure 8.3. Coalescent analysis of *Phytophthora* Ic clade species. The analyses were run a total of 5,000,000 simulations three separate times. *P. phaseoli* is basal in the clade.



andina ORF79, which appears to have a 4 amino acid deletion of phenylalanine, leucine, serine, and isoleucine. The high degree of similarity in gene order and function is needed for coding sequences for structural and functional genes. More unique genes that distinguish species within the clade are found in the nuclear genes that encode for host specialization, virulence, or pathogenicity.

The mitochondrial genomes of *Phytophthora* species in the Ic clade were highly similar, differing only by 672 polymorphisms in the coding regions. The gene content and order were both conserved among all five species of the Ic clade, indicating that the mitochondrial genomes of the clade are not highly divergent. Comparison of our data with the type II mitogenomes of *P. infestans* showed that *Phytophthora* species in the Ic clade more closely resemble the type I mitogenomes of *P. infestans*. The four remaining Ic clade species show none of the unique ORFs found among the type II haplotypes of *P. infestans* and these species also lack the 2 kb insertion sequence that is characteristic of type II haplotypes of *P. infestans* (Avila-Adame *et al.*, 2006 Carter *et al.*, 1990; Gavino and Fry 2002).

Four mitochondrial haplotypes (Ia, Ib, IIa and IIb) have been designated in modern populations of *P. infestans* using PCR and RFLP analysis of mitochondrial DNA (Carter *et al.*, 1990; Griffith and Shaw 1998). The remaining three extant mtDNA haplotypes of *P. infestans* were sequenced by Avila *et al.*, and mutations leading to the evolution of both type I and type II mitochondrial lineages were clearly identified (Avila-Adame *et al.*, 2006). It had previously been proposed that the Ib haplotype was the ancestral lineage of *P. infestans* and that the type II's evolved from the type I lineages (Gavino and Fry 2002; Goodwin *et al.*,

1994). However, Avila-Adame *et al.*, (2006) found that the type II mitochondrial lineages of *P. infestans* diverged earlier from a common ancestor than the type I haplotypes. Based on our data, the remaining members of the *Phytophthora* Ic clade species share an early common ancestor of *P. infestans* but diverged even earlier before the type II haplotypes of *P. infestans* diverged. Recently, a Bayesian phylogeny of *P. infestans* mitogenomes from modern and historic lineages has dated the estimated coalescence time of all the mitogenomes of *P. infestans* to 460-638 years (Martin et al 2014; Yoshida et al 2012). Thus the emergence of the other species in clade Ic must predate this estimated coalescent time.

Comparisons of the genomes' amino acid replacements suggest that each member of the Ic clade, including *P. andina*, is a distinct species and not a subspecies of another (Oliva *et al.*, 2010, Cardenas *et al.*, 2012). A particularly distinguishing feature of the *P. andina* genome is the presence of two indels in ORF79, a feature that can be used to distinguish *P. andina* from the other species that have a longer ORF79. This site may be used with other known regions to differentiate *P. andina* and *P. infestans* from one another. Creating a PCR primer specific to the *P. andina* ORF79 could enable a quick diagnostic tool for identification of this particular species in an infected plant. This indel is also present in the Ia mitochondrial lineage of *P. andina*, making this a feature unique to this species.

The mitochondrial genome genealogies suggests strongly that the Ic mitochondrial lineage of *P. andina* is a unique species and more ancient than the mitochondrial lineages of *P. infestans*. However, based on the mitochondrial genome sequences, the Ia lineage of *P. andina*, is more closely related to *P. infestans* than the Ic lineage. This has also been suggested by others using multilocus sequencing of nuclear and mitochondrial genes (Blair *et*

al., 2012, Goss *et al.*, (2011). The coalescent analysis of the mitochondrial genomes cannot rule out the hypothesis that the *P. andina* lineage arose via hybridization between *P. infestans* and another species (Goss et al 2011, Gomez *et al.*, 2007). The coalescent data shows that the *P. andina* Ia haplotype is more closely related to the type I haplotypes of *P. infestans*, but the Ic lineage is more closely related to *P. ipomoeae* and *P. mirabilis* and shares a common ancestor with these species. This hypothesis may also help to resolve the species tree estimates posited by Blair *et al.*, (2012), where *P. andina* “A” (Ia) continually forms clades with *P. infestans*, while *P. andina* “B” (Ic) generally formed a separate monophyletic lineage. Both lineages of *P. andina* contain the same mutations in Orf79. It is clear that the Ic lineage of *P. andina* shares a common ancestor with *P. infestans*.

It has been proposed that the Toluca Valley in Mexico is the center of origin for *P. infestans* and its close relatives *P. ipomoeae* and *P. mirabilis* (Gavino and Fry 2002; Grünwald and Flier 2005; Flier *et al.*, 2003). However, alternative data suggests the Andes of South America as the center of origin of *P. infestans* (Gómez-Alpizar *et al.*, 2007). The evolutionary history of the Ic clade inferred from the coalescent analyses presented here supports the latter hypothesis. *P. infestans*, *P. mirabilis*, *P. andina*, and *P. ipomoeae* coalesced into the same group, indicating that these four species share a common ancestor that must have been present together in the past. To date, *P. andina* has only been reported in the Andean region of South America. This suggests that the divergence of the *P. andina* Ic lineage from *P. infestans* occurred in the Andean region. Subsequently, *P. ipomoeae* and *P. mirabilis* are sister species that evolved more recently from a common ancestor of the Ic

lineage of *P. andina*. These data suggest that *P. mirabilis* and *P. ipomoeae*, two species currently reported only in Mexico, may also occur in the Andean region.

The host of *P. mirabilis*, *Mirabilis jalapa* and the host plant for *P. ipomoeae*, *Ipomoeae longipedunculata*, both originated from the Andean region of South America, and were transported to Mexico hundreds of years ago; neither are native to Mexico. One potential scenario includes the possibility that these *Phytophthora* species jumped hosts in Mexico from a wild *Solanum* host (Grünwald and Flier, 2005). An alternative possibility is that the entire Ic clade species evolved in South America on the hosts that were native there and were then moved to Mexico on infected plants. The later hypothesis is more likely since *P. andina* and *P. infestans* are sympatric in the Andean region and it is clear that *P. mirabilis* and *P. ipomoeae* diverged more recently from a common ancestor of the *P. andina* Ic lineage.

We cannot rule out the alternative hypothesis of the entire Ic clade evolving in Mexico. Although other members of the Ic clade have been found in Mexico, the hosts that they infect were brought to the region from South America. *P. andina* may be present in Mexico, but there are currently no data to support this hypothesis since *P. andina* has not been found in this region. Further surveys for *P. andina* in Mexico and elsewhere in Central and South America are warranted in addition to reciprocal surveys for *P. mirabilis* and *P. ipomoeae* in the Andean region. South America is also considered as the center of origin for the entire genus *Solanum*, a major host of *Phytophthora* species in the Ic clade (Hawkes 1990). Host speciation may have led to the evolution of the unique mitochondrial lineages of the Ic clade species of *Phytophthora*.

A study by Farrer *et al.*, (2011) on the pathogen *Batrachochytrium dendrobatidis*, demonstrated that multiple lineages of the fungus are being vectored between continents through the movement of the amphibian host. Here, they used whole genome sequences to determine global emergences of various lineages. A similar method, using the next generation sequencing of whole genomes of the Ic *Phytophthora* species, should be used in order to better characterize the evolutionary relationships of the clade and determine implications for future *Phytophthora* diseases on their respective hosts. Also, surveys conducted in both Mexico, Central, and South America are needed to resolve the center of origin debate for the clade.

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APPENDICES

APPENDIX A

Table S8.1. Annotation of mitochondrial genes of the Ic clade species of *Phytophthora*

	Species						Location
	<i>P. infestans</i>	<i>P. phaseoli</i>	<i>P. andina Ic</i>	<i>P. andina Ia</i>	<i>P. ipomoeae</i>	<i>P. mirabilis</i>	
Start	26	26	26	26	26	24	rnl
End	2679	2679	2679	2679	2679	2677	rnl
Start	2686	2686	2686	2686	2686	2684	trnN
End	2757	2757	2757	2757	2757	2755	trnN
Start	2770	2770	2770	2770	2770	2768	trnS (gcu)
End	2858	2858	2858	2858	2858	2856	trnS (gcu)
Start	2879	2879	2879	2879	2879	2877	trnM (cau)
End	2950	2950	2950	2950	2950	2948	trnM (cau)
Start	2994	2994	2994	2994	2994	2992	trnP
End	3068	3068	3068	3068	3068	3066	trnP
Start	3082	3082	3082	3082	3082	3080	trnM
End	3153	3153	3153	3153	3153	3151	trnM
Start	3171	3171	3171	3171	3171	3169	rpl14
End	3542	3542	3542	3542	3542	3540	rpl14
Start	3549	3549	3549	3549	3549	4547	rpl5
End	4082	4082	4082	4082	4082	4080	rpl5
Start	4090	4090	4090	4090	4090	1088	trnG (gcc)
End	4161	4161	4161	4161	4161	4159	trnG (gcc)
Start	4253	4253	4253	4253	4253	4251	trnG (ucc)
End	4324	4324	4324	4324	4324	4322	trnG (ucc)
Start	4330	4330	4330	4330	4330	4328	trnY
End	4413	4413	4413	4413	4413	4411	trnY
Start	5199	5210	5194	5194	5187	5209	Rns
End	6701	6712	6696	6696	6689	6711	Rns
Start	6736	6747	6731	6731	6724	6746	trnW
End	6807	6818	6802	6802	6795	6817	trnW

Table S8.1. Continued

Start	7002	7013	6983	6984	6988	7000	orf79
End	7241	7252	7210	7211	7227	7239	orf79
Start	7661	7651	7617	7618	7631	7596	cox2
End	8437	8427	8393	8394	8407	8372	cox2
Start	8453	8443	8409	8410	8423	8388	orf32
End	8551	8541	8507	8508	8521	8486	orf32
Start	8639	8624	8591	8596	8605	8572	cox1
End	10117	10102	10069	10074	10083	10050	cox1
Start	10266	10248	10215	10222	10230	10198	atp9
End	10493	10475	10442	10449	10457	10425	atp9
Start	10655	10633	10601	10609	10615	10586	nad9
End	11221	11199	11167	11175	11181	11152	nad9
Start	11282	11260	11228	11236	11242	11213	cob
End	12433	12411	12379	12387	12393	12364	cob
Start	12572	12542	12506	12513	12528	12496	atp1
End	14101	14071	14035	14042	14057	14025	atp1
Start	14182	14152	14166	14123	14137	14106	trnE
End	14253	14223	14187	14194	14208	14177	trnE
Start	14283	14253	14217	14224	14238	14207	nad4
End	15758	15728	15692	15699	15713	15682	nad4
Start	15781	15751	15715	15722	15736	15705	trnH
End	15853	15823	15787	15794	15808	15777	trnH
Start	15857	15827	15791	15798	15812	15781	orf142
End	16285	16255	16219	16226	16240	16209	orf142
Start	16295	16265	16229	16236	16250	16219	nad7
End	17473	17443	17407	17414	17428	17397	nad7
Start	17591	17559	17523	17530	17544	17515	nad2
End	19084	19052	19016	19023	19037	19008	nad2
Start	19091	19059	19023	19030	19044	19015	trnF
End	19164	19132	19096	19103	19117	19088	trnF

Table S8.1. Continued

Start	19183	19151	19115	19122	19136	19107	rps10
End	19509	19477	19441	19448	19462	19433	rps10
Start	19514	19482	19446	19453	19467	19438	trnR (gcg)
End	19587	19555	19519	19526	19540	19511	trnR (gcg)
Start	19598	19566	19530	19537	19551	19522	trnQ
End	19669	19637	19601	19608	19622	19593	trnQ
Start	19671	19639	19603	19610	19627	19595	trnI (gau)
End	19744	19712	19676	19683	19697	19668	trnI (gau)
Start	19747	19715	19679	19686	19700	19671	trnV
End	19819	19787	19751	19758	19772	19743	trnV
Start	19836	19804	19768	19775	19789	19760	rps12
End	20216	20184	20148	20155	20169	20140	rps12
Start	20146	20114	20078	20085	20099	20070	rps7
End	20619	20587	20551	20558	20572	20543	rps7
Start	20675	20643	20607	20614	20628	20599	cox3
End	21592	21560	21523	21531	21545	21516	cox3
Start	21620	21588	21552	21559	21573	21544	atp6
End	22339	22307	22271	22278	22292	22263	atp6
Start	22370	22338	22302	22309	22322	22294	trnD
End	22443	22411	22375	22382	22395	22367	trnD
Start	22480	22448	22412	22419	22432	22404	nad3
End	22833	22801	22765	22772	22785	22757	nad3
Start	23053	23031	22983	22989	22995	22877	nad5
End	25047	25025	24977	24983	24989	24871	nad5
Start	25091	25069	25021	25027	25033	24915	nad6
End	25801	25779	27571	25737	25743	25625	nad6
Start	25827	25804	25747	25753	25769	25651	trnR (ucu)
End	25899	25876	25819	25825	25841	25723	trnR (ucu)
Start	26064	36025	25985	25991	25985	25887	nad4L
End	26367	26327	26287	26293	26287	26189	nad4L

Table S8.1. Continued

Start	26370	26330	26290	26296	26290	26192	nad1
End	27350	27310	27270	27276	27270	27172	nad1
Start	27347	273007	27267	27273	27267	27169	nad11
End	29353	29313	29273	29279	29273	29175	nad11
Start	29438	29396	29356	29363	29356	29260	trnL (uag)
End	29520	29478	29438	29445	29438	29342	trnL (uag)
Start	29530	29488	29448	29455	29448	29352	trnL (uaa)
End	29613	29571	29531	29538	29531	29435	trnL (uaa)
Start	29633	29591	29551	29559	29551	29455	ymf16
End	30379	30337	30297	30305	30297	30201	ymf16
Start	30570	30526	30486	30496	30486	30390	trnC
End	30640	30596	30556	30566	30556	30460	trnC
Start	30652	30608	30568	30578	30568	30472	trnS (uga)
End	30736	30692	30652	30662	30652	30556	trnS (uga)
Start	30758	30712	30673	30684	30673	30577	rps11
End	31174	31128	31089	31100	31089	30991	rps11
Start	31187	31141	31102	31113	31102	31006	rps13
End	31600	31554	31515	31526	31515	31419	rps13
Start	31629	31583	31544	31555	31544	31448	rpl2
End	32435	32389	32350	32361	32350	32254	rpl2
Start	32439	32393	32354	32365	32354	32258	rps19
End	32672	32626	32587	32598	32587	32491	rps19
Start	32676	32630	32591	32602	32591	32495	rps3
End	33479	33433	33394	33405	33394	33298	rps3
Start	33482	33436	33397	33408	33397	33301	rpl16
End	33886	3384	33801	33812	33801	33705	rpl16
Start	33889	33843	33804	33815	33804	33708	trnI (cau)
End	33962	33916	33877	33888	33877	33781	trnI (cau)
Start	33983	33937	33898	33909	33898	33802	orf217
End	34636	34590	34551	34562	34551	34455	orf217

Table S8.1. Continued

Start	34698	34652	34613	34624	34613	34517	atp8
End	35090	35044	35005	35016	35005	34909	atp8
Start	35111	35066	35027	35037	35027	34931	trnK
End	35183	35138	35099	35109	35099	35003	trnK
Start	35187	35142	35103	35113	35103	35007	trnA
End	35259	35214	35175	35185	35175	35079	trnA
Start	35278	35233	35194	35204	35194	35098	rps14
End	35577	35532	35493	35503	35493	35397	rps14
Start	35591	35546	35509	35517	35507	35411	rps8
End	35971	35926	35889	35897	35887	35791	rps8
Start	35979	35934	35896	35905	35984	35799	rpl6
End	36584	36439	36501	36510	36499	36404	rpl6
Start	36591	36546	36508	36517	36506	36411	rps2
End	37181	37136	37098	37107	37096	37001	rps2
Start	37191	37146	37108	37117	37106	37011	rps4
End	37652	37607	37569	37578	37567	37472	rps4
Start	37655	37610	37572	37581	37570	37475	orf100
End	37957	37912	37874	37883	37872	37777	orf100

APPENDIX B

Table S8.2. Polymorphisms between Ic clade *Phytophthora* species based on *P. infestans* Ib mt genome.

Polymorphism mutation ^a	<i>P. infestans</i>	<i>P. mirabilis</i>	<i>P. andina Ic</i>	<i>P. andina Ia</i>	<i>P. ipomoeae</i>	<i>P. phaseoli</i>	Gene
CCTCCC	174	172	174	174	174	174	rnl
GGAGGG	200	198	200	200	200	200	rnl
GGAGGG	231	229	231	231	231	231	rnl
GGGGGA	353	351	353	353	353	353	rnl
TTTTCT	373	371	373	373	373	373	rnl
TTTTTA	514	512	514	514	514	514	rnl
GGAGGG	1002	1000	1002	1002	1002	1002	rnl
CCTCCC	1090	1088	1090	1090	1090	1090	rnl
AAGAAA	1114	1112	1114	1114	1114	1114	rnl
TTATAT	1140	1138	1140	1140	1140	1140	rnl
CCCCCT	1272	1270	1272	1272	1272	1272	rnl
TAATTT	1548	1546	1548	1548	1548	1548	rnl
ATTAAA	1551	1549	1551	1551	1551	1551	rnl
TAATTT	1554	1552	1554	1554	1554	1554	rnl
GTTGTG	2022	2020	2022	2022	2022	2022	rnl
CCCCCT	2089	2087	2089	2089	2089	2089	rnl
GGGGGA	2105	2103	2105	2105	2105	2105	rnl
AGAAAA	2111	2109	2111	2111	2111	2111	rnl
ATAAAA	2572	2570	2572	2572	2572	2572	rnl
TCCTCT	2701	2699	2701	2701	2701	2701	trnN
CAACAA	2728	2726	2728	2728	2728	2728	trnN
GGAGGG	3000	2998	3000	3000	3000	3000	trnP
CCTCCC	3203	3201	3203	3203	3203	3203	rpl14
AAAAAC	3340	3338	3340	3340	3340	3340	rpl14
CTTCTT	3365	3363	3365	3365	3365	3365	rpl14
TAATAA	3404	3402	3404	3404	3404	3404	rpl14
CCTCCT	3470	3468	3470	3470	3470	3470	rpl14
GGAGGG	3594	3592	3594	3594	3594	3594	rpl5
CCCCCT	3615	3613	3615	3615	3615	3615	rpl5
AAAAGA	3791	3789	3791	3791	3791	3791	rpl5
CAACCA	3853	3851	3853	3853	3853	3853	rpl5
AAAAAG	3912	3910	3912	3912	3912	3912	rpl5
AAAAAG	3913	3911	3913	3913	3913	3913	rpl5
TTTTCT	3917	3915	3917	3917	3917	3917	rpl5
CCCCTT	3944	3942	3944	3944	3944	3944	rpl5
CCCCCT	3974	3972	3974	3974	3974	3974	rpl5
TTTTTC	3995	3993	3995	3995	3995	3995	rpl5
AAAAGG	4147	4145	4147	4147	4147	4147	trnG (gcc)
GAGGGG	4397	4395	4397	4397	4397	4397	trnY

Table S8.2. Continued

AGAAAG	5278	5276	5278	5278	5278	5278	rns
CCCCCT	5337	5347	5332	5332	5325	5069	rns
ACCACC	5427	5437	5422	5422	5415	5438	rns
TTGTTT	5778	5788	5773	5773	5766	5789	rns
AACAAA	5853	5863	5848	5848	5841	5864	rns
CTTTTT	6086	6096	6081	6081	6074	6097	rns
AAAAAG	6146	6156	6141	6141	6134	6157	rns
AAAAAG	6248	6258	6243	6243	6236	6259	rns
ATAAAA	6250	6260	6245	6245	6238	6261	rns
CCCCCT	6265	6275	6260	6260	6253	6276	rns
AAAAGA	6363	6373	6358	6358	6351	6374	rns
GGAGGG	6148	6428	6413	6413	6406	6429	rns
AAAAAG	6596	6606	6591	6591	6584	6607	rns
CCCCCT	6629	6639	6624	6624	6617	6640	rns
AAAAAG	6762	6772	6757	6757	6750	6773	trnW
TATTTT	7023	7021	7004	7005	7009	7034	orf79
CTTTTC	7028	7026	7009	7010	7014	7039	orf79
TTCCAT	7034	7032	7015	7016	7020	7045	orf79
CCTCCC	7119	7099	7070	7071	7087	7112	orf79
AAAAAC							orf79
CTTCTT							orf79
TTTTAT							orf79
TCCTCC							orf79
TTTTTC							orf79
AAGAGA							orf79
CCTCTC							orf79
GGGGGA							orf79
GAAAAG							orf79
CCCCTC	7795	7712	7733	7734	7747	7767	cox2
GAAGAA	7831	7748	7769	7770	7783	7803	cox2
GGGGAG	7871	7788	7809	7810	7823	7843	cox2
CCCCCT	7892	7809	7830	7831	7844	7864	cox2
TCCTCC	7962	7879	7900	7901	7914	7934	cox2
CCCCTC	7981	7898	7919	7920	7933	7953	cox2
GGGGGA	7984	7901	7922	7923	7936	7956	cox2
GGGGAG	8167	8084	8105	8106	8119	8137	cox2
TTTTTC	8176	8093	8114	8115	8128	8148	cox2
GGAGGG	8207	8124	8145	8146	8159	8179	cox2
AGGAGG	8224	8141	8162	8163	8176	8196	cox2
ATAAAA	8239	8156	8117	8118	8191	8211	cox2
GAAGAA	8251	8168	8189	8189	8203	8223	cox2
TCTTTT	8278	8195	8216	8217	8230	8250	cox2
TTTTCT	8284	8201	8222	8223	8236	8256	cox2
GAGGGG	8441	8358	8379	8380	8393	8413	cox2

Table S8.2. Continued

GAGGGG	8450	8367	8388	8389	8402	8422	cox2
TTTTGT	8505	8422	8443	8444	8457	8477	orf32
CCACCC	8560	8477	8498	8499	8512	8532	orf32
TTTTTA	8677	8592	8611	8616	8625	8644	cox1
AAAAAC	8678	8593	8612	8617	8626	8645	cox1
AAAAGA	8797	8712	8731	8736	8745	8764	cox1
CCCCAC	8908	8823	8842	8847	8856	8875	cox1
AGGAGG	8390	8845	8864	8869	8878	8897	cox1
TTTTTG	8953	8868	8887	8892	8901	8920	cox1
AAAAAG	8998	8913	8932	8937	8946	8965	cox1
AAAAGA	9064	8979	8998	9003	9012	9031	cox1
TTTTGT	9109	9024	9043	9048	9057	9067	cox1
TTCTTT	9224	9139	9158	9163	9172	9191	cox1
CCCCTC	9287	9202	9221	9226	9235	9254	cox1
AGAAAA	9289	9205	9223	9228	9237	9256	cox1
CCCCCT	9331	9246	9265	9270	9279	9298	cox1
TCTTTT	9343	9258	9277	9282	9291	9310	cox1
AAAACA	9367	9282	9301	9306	9315	9334	cox1
CTTCTT	9383	9298	9317	9322	9331	9350	cox1
AAAAAG	9430	9345	9364	9369	9378	9397	cox1
CTTCTT	9445	9360	9379	9384	9393	9412	cox1
ACAAAA	9520	9435	9454	9459	9468	9487	cox1
CCCCCT	9527	9442	9461	9466	9475	9494	cox1
AAAACA	9535	9450	9469	9474	9483	9502	cox1
GGAGGG	9619	9534	9553	9558	9567	9586	cox1
ACAAAA	9625	9540	9559	9564	9573	9592	cox1
GGGGAG	9628	9543	9562	9567	9576	9595	cox1
CCCCTC	9733	9648	9667	9672	9681	9700	cox1
CCCCCT	9750	9665	9684	9689	9698	9717	cox1
GTTGTT	9757	9672	9691	9696	9705	9724	cox1
AAAAAG	9778	9693	9712	9717	9726	9745	cox1
CCCCCT	9790	9705	9724	9729	9738	9757	cox1
TTTTAT	9832	9747	9766	9771	9780	9799	cox1
TTCTCC	9916	9831	9850	9855	9864	9883	cox1
CCCCCA	9951	9866	9885	9890	9899	9918	cox1
CTTCTC	9952	9867	9886	9891	9900	9919	cox1
GAAGAG	9982	9897	9916	9921	9930	9949	cox1
AAAAAC	9992	9907	9926	9931	9940	9959	cox1
TATTTT	10003	9918	9937	9942	9951	9970	cox1
TTTTCT	10009	9924	9943	9948	9957	9976	cox1
TTTCTT				10322			atp9
CCCTCC				10410			atp9
TTTTTC	10790	10703	10718	10726	10732	10750	nad9
TTTTTA	10801	10714	10729	10737	10743	10761	nad9

Table S8.2. Continued

GGAGGG	10927	10840	10855	10863	10869	10887	nad9
CCCCCT	10938	10851	10866	10874	10880	10898	nad9
AAAACA	10939	10852	10867	10875	10881	10899	nad9
GGTGGG	10988	10901	10916	10924	10930	10948	nad9
AAAAGG	11005	10918	10933	10941	10947	10965	nad9
TTTTTC	11164	11077	11092	11100	11106	11124	nad9
GAGGGG	11420	11333	11348	11356	11362	11380	cob
CCTCCC	11470	11383	11398	11406	11412	11430	cob
ACAAAA	11531	11444	11459	11467	11473	11491	cob
TTTTCT	11562	11475	11490	11498	11504	11522	cob
GGGGGA	11570	11483	11498	11506	11512	11530	cob
AGCAGG	11579	11492	11507	11515	11521	11539	cob
AAAACA	11581	11494	11509	11517	11523	11541	cob
CCCCTC	11619	11532	11547	11555	11561	11579	cob
AGGGGG	11623	11536	11551	11559	11565	11583	cob
AAAAAC	11633	11546	11561	11569	11575	11593	cob
TTTTCT	11743	11656	11671	11679	11685	11703	cob
CCCCCT	11758	11671	11686	11694	11700	11718	cob
TTTTTC	11857	11770	11785	11793	11799	11817	cob
AAAAGA	11899	11812	11827	11835	11841	11859	cob
CCCCTC	11989	11902	11917	11925	11931	11949	cob
CTTCTT	12016	11929	11944	11952	11958	11976	cob
AAAATA	12076	11989	12004	12012	12018	12036	cob
CTTCTT	12184	12097	12112	12120	12126	12144	cob
TTTTGT	12188	12101	12116	12124	12130	12148	cob
CCCCCT	12223	12136	12151	12159	12165	12183	cob
TCTTCT	12235	12148	12163	12171	12177	12195	cob
TTTTAT	12349	12262	12277	12285	12291	12309	cob
TTCTTT	12361	12274	12289	12297	12303	12321	cob
AAAAAG	12704	12610	12620	12627	12642	12656	atp1
GGGGGA	12716	12622	12632	12639	12654	12668	atp1
CTTCTT	12740	12646	12656	12663	12678	12692	atp1
GGAGGG	12741	12647	12657	12664	12679	12693	atp1
CCCCTC	12808	12714	12724	12731	12746	12760	atp1
TTTTTC	12866	12772	12782	12789	12804	12818	atp1
CTTCTT	12914	12820	12830	12837	12852	12866	atp1
GGAGGA	12917	12823	12833	12840	12855	12869	atp1
GAAGAA	13085	12991	13001	13008	13023	13037	atp1
AAAACA	13121	13027	13037	13044	13059	13073	atp1
AAAAAG	13127	13033	13043	13050	13065	13079	atp1
GGGGGA	13220	13126	13136	13143	13158	13172	atp1
TTTTTC	13343	13249	13259	13266	13281	13295	atp1
AAAAGA	13346	13252	13262	13269	13284	13298	atp1
TTTTTC	13406	13312	13322	13329	13344	13358	atp1

Table S8.2. Continued

TCCTCA	13409	13315	13325	13332	13347	13361	atp1
TCTTTT	13463	13369	13379	13386	13401	13415	atp1
AGGAGG	13526	13432	13442	13449	13464	13478	atp1
GAGGGG	13542	13448	13458	13465	13480	13494	atp1
GGGGTG	13607	13513	13523	13530	13545	13559	atp1
TAATAA	13619	13525	13535	13542	13557	13571	atp1
GAGGAA	13649	13555	13565	13572	13587	13601	atp1
ATGATT	13724	13630	13640	13647	13662	13676	atp1
AAAAGA	13795	13701	13711	13718	13733	13747	atp1
AGGAGA	13853	13759	13769	13776	13791	13805	atp1
CCTCCC	13860	13766	13776	13783	13798	13812	atp1
TTCTTT	13953	13859	13869	13876	13891	13905	atp1
GGGGAG	13982	13888	13898	13905	13920	13934	atp1
AAAACA	14012	13918	13928	13935	13950	13964	atp1
AAAAAG	14021	13927	13937	13942	13959	13973	atp1
GGGGAG	14241	14147	14157	14164	14178	14193	trnE
GGGGGA	14322	14228	14238	14245	14259	14274	nad4
GGGGGA	14397	14303	14313	14320	14334	14349	nad4
TTTTCT	14493	14399	14409	14416	14430	14445	nad4
CCACCC	14522	14428	14438	14445	14459	14474	nad4
GGGGGA	14547	14453	14463	14470	14484	14499	nad4
TTCTTT	14601	14507	14517	14524	14538	14553	nad4
CTTTTT	14622	14528	14538	14545	14559	14574	nad4
GGGGTT	14694	14600	14610	14617	14631	14646	nad4
AAAAAG	14760	14666	14676	14683	14697	14712	nad4
TTGTTT	14928	14834	14844	14851	14865	14880	nad4
TCTTTT	14972	14878	14888	14895	14909	14924	nad4
CTCCCA	14973	14879	14889	14896	14910	14925	nad4
AAAAGA	15012	14918	14928	14925	14949	14964	nad4
TTTTTC	15042	14948	14958	14965	14979	14994	nad4
AACAAA	15054	14960	14970	14977	14991	15006	nad4
CCCCCA	15102	15008	15018	15025	15039	15054	nad4
GGAGGG	15168	15074	15084	15091	15105	15120	nad4
TTCTTT	15301	15207	15217	15224	15238	15253	nad4
TTTTCT	15333	15239	15249	15256	15270	15285	nad4
GGGGGA	15381	15287	15297	15304	15318	15333	nad4
TTTTGT	15425	15331	15341	15348	15362	15377	nad4
AGGAGA	15495	15401	15411	15418	15432	15447	nad4
CCCCCT	15725	15631	15641	15648	15662	15677	nad4
CCCCCT	15813	15719	15729	15737	15750	15765	trnH
CTTCTT	15914	15820	15830	15837	15851	15866	orf142
GAAGAA	16016	15922	15932	15939	15953	15968	orf142
CCCCCT	16057	15963	15973	15980	15994	16009	orf142
AGGAGG	16116	16022	16032	16039	16053	16068	orf142

Table S8.2. Continued

CCCCCT	16160	16066	16076	16083	16097	16112	orf142
AAAAAT	16180	16086	16096	16103	16117	16132	orf142
GAGGGG	16190	16096	16106	16113	16127	16142	orf142
CCTCCC	16298	16204	16214	16221	16235	16250	orf142
AAAACA	16511	16417	16427	16434	16448	16463	nad7
ATAAAA	16547	16453	16463	16470	16484	16499	nad7
GGGGGA	16616	16522	16532	16539	16553	16568	nad7
ACAAAA	16703	16609	16619	16626	16640	16655	nad7
TTTTCT	16778	16684	16694	16701	16715	16730	nad7
AAAAGA	16811	16717	16727	16734	16748	16763	nad7
CTTCTC	16862	16768	16778	16785	16799	16814	nad7
CCCCCT	16928	16834	16844	16851	16865	16880	nad7
AGGAGT	16946	16852	16862	16869	16883	16898	nad7
TTGTTT	16973	16879	16889	16896	16910	16925	nad7
CCCCCA	17012	16918	16928	16935	16949	16964	nad7
TTTTTC	17018	16924	16934	16941	16955	16970	nad7
TTTTTG	17033	16939	16949	16956	16970	16985	nad7
TTTTCT	17096	17002	17012	17019	17033	17048	nad7
GGGGGT	17194	17100	17110	17117	17131	17146	nad7
AAAAAC	17204	17110	17120	17127	17141	17156	nad7
TTTTTC	17210	17116	17126	17133	17147	17162	nad7
AGAAAA	17213	17119	17129	17136	17150	17165	nad7
GGGGGA	17216	17122	17132	17139	17153	17168	nad7
AACAAA	17372	17278	17288	17295	17309	17324	nad7
TGTTTT	17390	17296	17306	17313	17327	17342	nad7
AAAAAG	17404	17310	17320	17327	17341	17356	nad7
AAGAAA	17441	17347	17357	17364	17378	17393	nad7
AGGGGG	17900	17806	17814	17821	17835	17850	nad2
GGGGTG	18035	17941	17949	17956	17970	17985	nad2
TTTTCT	18100	18006	18014	18021	18035	18050	nad2
GGGGGA	18179	18085	18093	18100	18114	18129	nad2
GGTGGG	18311	18217	18225	18232	18246	18261	nad2
GGGGGA	18322	18228	18236	18243	18257	18272	nad2
GTTGTT	18397	18303	18311	18318	18332	18347	nad2
AATAAA	18449	18355	18363	18370	18384	18399	nad2
AAAAGA	18505	18411	18419	18426	18440	18455	nad2
CCTCCC	18520	18426	18434	18441	18455	18470	nad2
GAAGAA	18529	18435	18443	18450	18464	18479	nad2
AAAAAG	18538	18444	18452	18459	18473	18488	nad2
TCTTTT	18565	18471	18479	18486	18500	18515	nad2
CCTCCC	18644	18550	18558	18565	18579	18594	nad2
AAAAGA	18712	18618	18626	18633	18647	18662	nad2
CTACCC	18805	18711	18719	18726	18740	18755	nad2
CAACAA	18841	18747	18755	18766	18776	18791	nad2

Table S8.2. Continued

CTTCTT	18883	18789	18797	18804	18818	18833	nad2
GGGGGA	19006	18912	18920	18927	18941	18956	nad2
TTTTTC	19014	18920	18928	18935	18949	18964	nad2
TTTTTA	19021	18927	18935	18946	18956	18971	nad2
TTTTTC	19037	18943	18951	18958	18972	18987	nad2
ACCACC	19047	18953	18961	18968	18982	18997	nad2
GGGGGA	19064	18970	18978	18985	18999	19014	nad2
AGAAAA	19079	18985	18993	19000	19014	19029	nad2
TCCTCC	19111	19017	19025	19032	19046	19061	trnF
AAAACA	19277	19183	19191	19198	19212	19227	rps10
CCCCCT	19322	19228	19236	19243	19257	19272	rps10
AGAAAA	19453	19359	19367	19374	19388	19403	rps10
GAGGGG	19558	19464	19472	19479	19493	19508	trnR
GGGGGA	19589	19495	19503	19510	19524	19539	trnR
TTTTTA	19865	19771	19779	19786	19800	19815	rps12
CTTCTT	19928	19834	19842	19849	19863	19878	rps12
AACAAA	19985	19891	19899	19906	19920	19935	rps12
GGGGGA	19994	19900	19908	19915	19929	19944	rps12
TTCTTT	20027	19933	19941	19948	19962	19977	rps12
TTTTTC	20028	19934	19942	19949	19963	19978	rps12
TTTTTA	20034	19940	19948	19955	19969	19984	rps12
TCCTCT	20036	19942	19950	19957	19971	19986	rps12
GAAGAA	20039	19945	19953	19960	19974	19989	rps12
GAAGAA	20045	19951	19959	19966	19980	19995	rps12
AGAAGA	20054	19960	19968	19975	19989	20004	rps12
TTTTCT	20060	19966	19974	19981	19995	20010	rps12
GGAGGG	20063	19969	19977	19984	19998	20013	rps12
AAAAAT	20072	19978	19986	19993	20007	20022	rps12
GGGGGA	20087	19993	20001	20008	20022	20037	rps12
AGGAGG	20108	20014	20022	20029	20043	20058	rps12
CCCCCA	20154	20060	20068	20075	20089	20104	rps12
CCCCCT	20189	20095	20103	20110	20124	20139	rps12
AAAAAG	20198	20104	20112	20119	20133	20148	rps12
CCCCCT	20189	20095	20103	20110	20124	20139	rps7
AAAAAG	20198	20104	20112	20119	20133	20148	rps7
TTTTTG	20385	20291	20299	20306	20320	20335	rps7
CCCCCT	20406	20312	20320	20327	20341	20356	rps7
GGAGGG	20440	20346	20354	20361	20375	20390	rps7
GGGGGA	20534	20440	20448	50455	20469	20484	rps7
CCCCAC	20729	20635	20643	20650	20664	20679	cox3
CCCCTC	20803	20709	20717	20724	20738	20753	cox3
GGGGAG	20818	20724	20732	20739	20753	20768	cox3
TTTTTG	20836	20742	20750	20757	20771	20786	cox3
AAAAAC	20896	20802	20810	20817	20831	20846	cox3

Table S8.2. Continued

GGGGGA	20906	20812	20820	20827	20841	20856	cox3
ACCACT	20920	20826	20834	20841	20855	20870	cox3
TCCTCC	21127	21033	21041	21048	21062	21077	cox3
CAACAA	21145	21051	21059	21066	21080	21095	cox3
GGGGGA	21187	21093	21101	21108	21122	21137	cox3
TTTTTC	21274	21180	21188	21195	21209	21224	cox3
AAGAAA	21511	21417	21425	21432	21446	21461	cox3
CCCCCT	21514	21420	21428	21435	21449	21464	cox3
CCTCCT	21520	21426	21434	21441	21455	21470	cox3
AACAAA	21583	21489	21497	21504	21518	21533	cox3
TTTTCT	21649	21555	21563	21570	21584	21599	atp6
GGGGGA	21692	21598	21606	21613	21627	21642	atp6
GGAGGG	21725	21631	21639	21646	21660	21675	atp6
TTTTTC	21808	21714	21722	21729	21743	21758	atp6
TTTTTA	21841	21747	21755	21762	21776	21791	atp6
CCCCCT	21849	21755	21763	21770	21784	21799	atp6
CCCCCT	21928	21834	21842	21849	21863	21878	atp6
TCTTTT	21943	21849	21857	21864	21878	21893	atp6
CCTCCC	21978	21884	21892	21899	21913	21928	atp6
AGAAAA	22081	21987	21995	22002	22016	22031	atp6
TTTTCT	22111	22017	22025	22032	22046	22061	atp6
TGGTTG	22129	22035	22043	22050	22064	22079	atp6
ACCACC	22133	22039	22047	22054	22068	22083	atp6
CCTCCT	22216	22122	22130	22137	22151	22166	atp6
GGGGGA	22327	22233	22241	22248	22262	22277	atp6
GGGGGA	22445	22351	22359	22366	22379	22395	trnD
CCCCCT	22514	22420	22428	22435	22448	22464	nad3
AAAAAG	22614	22520	22528	22535	22548	22564	nad3
GGAGGG	22648	22554	22562	22569	22582	22598	nad3
CCCCCT	22707	22613	22621	22628	22641	22657	nad3
AAAAAG	22713	22619	22627	22634	22647	22663	nad3
GGGGGA	22726	22632	22640	22647	22660	22676	nad3
TTTTTA	22779	22685	22693	22700	22713	22729	nad3
AAAAAG	23083	22889	22995	23001	23007	23043	nad5
CCTCCC	23084	22890	22996	23002	23008	23044	nad5
GGTGGG	23157	22963	23069	23075	23081	23117	nad5
AAAAAC	23194	23000	23106	23112	23118	23154	nad5
ACAAAA	23248	23054	23160	23166	23172	23208	nad5
TCCTCC	23310	23116	23222	23228	23234	23270	nad5
GTTGTT	23343	23149	23255	23261	23267	23303	nad5
CCTCCC	23391	23197	23303	23309	23315	23351	nad5
TAATAG	23406	23212	23318	23324	23330	23366	nad5
AGAAAA	23490	23296	23402	23408	23414	23450	nad5
TTTTCT	23532	23338	23444	23450	23456	23492	nad5

Table S8.2. Continued

AGAAAA	23550	23355	23462	23468	23474	23510	nad5
GGAGGG	23588	23394	23500	23506	23512	23548	nad5
CTTCTC	23599	23405	23511	23517	23523	23559	nad5
TTTTTC	23655	23461	23567	23573	23579	23615	nad5
AAAAAG	23686	23492	23598	23604	23610	23646	nad5
GAAGAA	23760	23566	23672	23678	23684	23720	nad5
CCCCCT	23800	23606	23712	23718	23724	23760	nad5
ACAAAA	23850	23656	23762	23768	23774	23810	nad5
GGGGGT	23901	23707	23813	23819	23825	23861	nad5
AAGAAA	23907	23713	23819	23825	23831	23867	nad5
GGGGAA	23974	23780	23886	23892	23898	23934	nad5
AAAAAG	23979	23785	23891	23897	23903	23939	nad5
TTTTTC	24024	23830	23936	23942	23948	23984	nad5
AAAAAG	24078	23884	23990	23996	24002	24038	nad5
GGGGAA	24182	23988	24094	24100	24106	24142	nad5
ACAAAA	24190	23996	24102	24108	24114	24150	nad5
ACCACC	24198	24004	24110	24116	24122	24158	nad5
CCCCCT	24282	24088	24194	24200	24206	24242	nad5
ACAAAA	24297	24103	24209	24215	24221	24257	nad5
TTTTCT	24631	24437	24543	24549	24555	24591	nad5
TTTTCT	24726	24532	24638	24634	24650	24686	nad5
AAAAAC	24754	24560	24666	24672	24678	24714	nad5
AGAAAA	24790	24596	24702	27408	24714	24750	nad5
AAAAGA	24811	24617	24723	24729	24735	24771	nad5
TCCTCC	24864	24670	24776	27482	24788	24824	nad5
TTTTCT	24892	24698	24804	24810	24816	24852	nad5
TTTTTC	25038	24844	24950	24956	24962	24998	nad5
CCTCCC	25203	25009	25115	25121	25127	25163	nad6
CCTCCC	25227	25033	25139	25145	25151	25187	nad6
TATTTT	25253	25059	25165	25171	25177	25213	nad6
AAGAAA	25300	25106	25212	25218	25224	25260	nad6
CCTCCC	25303	25109	25215	25221	25227	25263	nad6
GGAGGG	25349	25155	25261	25267	25273	25309	nad6
AAGAAA	25465	25271	25377	25383	25389	25425	nad6
GTTGTC	25543	25349	25455	25461	25467	25503	nad6
AAAACA	25627	25433	25539	25545	25551	25587	nad6
CCCCCT	25693	25499	25605	25611	25617	25653	nad6
GGAGGG	26137	25941	26039	26045	26039	26079	nad4L
TTTTTA	26218	26022	26120	26126	26120	26160	nad4L
AAAAAG	26271	26075	26173	26179	26173	26213	nad4L
TTTTAT	26364	26168	26266	26272	26266	26306	nad4L
AAAAAG	26421	26225	26323	26329	26323	26363	nad1
CCCCCA	26502	26306	26404	26410	26404	26444	nad1
CCCCCT	26549	26353	26451	26457	26451	26491	nad1

Table S8.2. Continued

TTTTCT	26550	26354	26452	26458	26452	26492	nad1
TTTTTA	26574	26378	26476	26482	26476	26516	nad1
CCTCTT	26709	26513	26611	26617	26611	26651	nad1
ACCACG	26730	26534	26632	26638	26632	26672	nad1
AAAACA	26748	26552	26650	26656	26650	26690	nad1
TTCTTT	26877	26681	26779	26785	26779	26819	nad1
GGGGGA	26892	26696	26794	26800	26794	26834	nad1
TTTTAT	26916	26720	26818	26824	26818	26858	nad1
AAAAAG	26919	26723	26821	26827	26821	26861	nad1
TTTTGT	26973	26777	26875	26881	26875	26915	nad1
AAAAGA	27000	26804	26902	26908	26902	26942	nad1
CCCCCT	27056	26860	26958	26964	26958	26998	nad1
AGAAAA	27069	26873	26971	26977	26971	27011	nad1
AAAAGA	27084	26888	26986	26992	26986	27026	nad1
CTTCTT	27219	27023	27121	27127	27121	27161	nad1
CCTCCT	27284	27088	27186	27192	27186	27226	nad1
TTTTGT	27333	27137	27235	27241	27235	27275	nad1
TTTTCT	27377	27181	27279	27285	27279	27319	nad11
TTCTTT	27461	27265	27363	27369	27363	27403	nad11
AAAAAG	27560	27364	27462	27468	27462	27502	nad11
CCCCCT	27603	27407	27505	27511	27505	27545	nad11
GGGGAG	27656	27460	27558	27564	27558	27598	nad11
AAATAA	27689	27493	27591	27597	27591	27631	nad11
AAAATA	27703	27507	27605	27611	27605	27645	nad11
AAAAGA	27781	27585	27683	27689	27683	27723	nad11
GGAGGG	27816	27620	27718	27724	27718	27758	nad11
TTTTGT	27828	27632	27730	27736	27730	27770	nad11
AGGAGG	27862	27666	27764	27770	27764	27804	nad11
GTGGGG	27886	27690	27788	27794	27788	27828	nad11
AAAAAG	27942	27746	27844	27850	27844	27884	nad11
CCACCC	28051	27855	27953	27959	27953	27993	nad11
GAGGGA	28142	27946	28044	28050	28044	28084	nad11
CTCCCC	28181	27985	28083	28089	28083	28123	nad11
CTCCCC	28240	28044	28142	28148	28142	28182	nad11
CCCCCT	28246	28050	28148	28154	28148	28188	nad11
AAAGAA	28247	28051	28149	28155	28149	28189	nad11
TTTTTC	28249	28053	28151	28157	28151	28191	nad11
TTTTTG	28280	28084	28182	28188	28182	28222	nad11
AAAAAG	28326	28130	28228	28234	28228	28268	nad11
AAAAAG	28372	28175	28273	28279	28273	28313	nad11
TTTTTG	28412	28216	28314	28320	28314	28354	nad11
AAAAAG	28436	28240	28338	28344	28338	28378	nad11
TTCTTT	28515	28319	28417	28423	28417	28457	nad11
TTTTCT	28530	28334	28432	28438	28432	28472	nad11

Table S8.2. Continued

CCCCCT	28534	28338	28436	28442	28436	28476	nad11
AAAAAG	28550	28354	28452	28458	28452	28492	nad11
AGAAAA	28583	28387	28485	28491	28485	28525	nad11
AGGAGA	28658	28462	28560	28566	28560	28600	nad11
AGGAGA	28697	28501	28599	28605	28599	28639	nad11
CTCCCC	28766	28570	28668	28674	28668	28708	nad11
AGGATA	28823	28627	28725	28731	28725	28765	nad11
AAAAGA	28844	28648	28746	28752	28746	28786	nad11
TGTTGT	28868	28672	28770	28776	28770	28810	nad11
GGGGGA	28905	28709	28807	28813	28807	28847	nad11
AAAAGA	28928	28732	28830	28836	28830	28870	nad11
GGGGAG	28970	28774	28872	28878	28872	28912	nad11
CCTCCC	28987	28791	28889	28895	28889	28929	nad11
AAAAAG	29086	28890	28988	28994	28988	29028	nad11
GGGGGA	29101	28905	29003	29009	29003	29043	nad11
GGGGGA	29170	28974	29072	29078	29072	29112	nad11
TTTTTG	29198	29002	29100	29106	29100	29140	nad11
AGGAGG	29222	29026	29124	29130	29124	29164	nad11
TTTTCT	29246	29050	29148	29154	29148	29188	nad11
TTTTAT	29290	29094	29192	29198	29192	29232	nad11
AAAACA	29296	29100	29198	29204	29198	29238	nad11
TTTTGT	29298	29102	29200	29206	29200	29240	nad11
GGAGGG	29307	29111	29209	29215	29209	29249	nad11
TCTTTT	29472	29276	29372	29379	29372	29412	trnL(uag)
TCTTTT	29509	29313	29409	29416	29409	29449	trnL(uag)
CCCCCT	29512	29316	29412	29419	29412	29452	trnL(uag)
AAAAAG	29523	29327	29423	29430	29423	29463	trnL(uag)
GTTGTT	29578	29382	29478	29485	29478	29518	trnL(uaa)
CTTCTT	29678	29482	29578	29586	29578	29618	ymf16
ACCACC	29747	29551	29647	29655	29647	29687	ymf16
TCTTTT	29854	29658	29754	29762	29754	29794	ymf16
TATTTT	29991	29795	29891	29899	29891	29931	ymf16
TTTTTC	30020	29824	29920	29928	29920	29960	ymf16
CACCCC	30030	29834	29930	29938	29930	29970	ymf16
GAAGAA	30035	29839	29935	29943	29935	29975	ymf16
GGGGGA	30053	29857	29953	29961	29953	29993	ymf16
TAATAA	30079	29883	29979	29987	29979	30019	ymf16
AAAAAG	30085	29889	29985	29993	29985	30025	ymf16
CCCCCT	30118	29922	30018	30026	30018	30058	ymf16
CAACAA	30160	29964	30060	20068	30060	30100	ymf16
GAAGAA	30373	31077	30273	30281	30273	30313	ymf16
TTTTTC	30393	31097	30293	30301	30293	30333	ymf16
TTTTTC	30603	30405	30501	30511	30501	30541	trnC
CCCCCT	30748	30550	30646	30656	30646	30686	trnS

Table S8.2. Continued

AAGAAA	30851	30652	30748	30759	30748	30787	rps11
CTTCTT	30869	30670	30766	30777	30766	30805	rps11
GACGGG	30896	30697	30793	30804	30793	30832	rps11
CCCCCT	31019	30820	30916	30927	30916	30955	rps11
GGTGGG	31042	30843	30939	30950	30939	30978	rps11
AAAAAT	31121	30922	31018	31029	31018	31057	rps11
TTTTAT	31140	30941	31037	31048	31037	31076	rps11
CTCCCC	31147	30948	31044	31055	31044	31083	rps11
GGGGGA	31249	31050	31146	31157	31146	31185	rps13
CTTCTT	31262	31063	31159	31170	31159	31198	rps13
ACCACC	31292	31093	31189	31200	31189	31228	rps13
TGTTTT	31297	31098	31194	31205	31194	31233	rps13
TTCTTT	31322	31123	31219	21330	31219	31258	rps13
GGGGGA	31328	31129	31225	31236	31225	31264	rps13
ACAAAA	31400	31201	31297	31308	31297	31336	rps13
GTGGGG	31401	31202	31298	31309	31298	31337	rps13
CCACCC	31415	31216	31312	31323	31312	31351	rps13
CCTCCC	31538	31339	31435	31446	31435	31474	rps13
GGAGAA	31550	31351	31447	31458	31447	31486	rps13
CCACTT	31558	31359	31455	31466	31455	31494	rps13
AGAAAA	31573	31374	31470	31481	31470	31509	rps13
AAAAAC	31652	31453	31549	31560	31549	31588	rpl2
AAGAAA	31712	31513	31609	31620	31609	31648	rpl2
GGGGGA	31801	31602	31698	31709	31698	31737	rpl2
CAACAA	31943	31744	31840	31851	31840	31879	rpl2
GATGGG	32027	31828	31924	31935	31924	31963	rpl2
CCTCTT	32045	31846	31942	31953	31942	31981	rpl2
CCCCCT	32051	31852	31948	31959	31948	31987	rpl2
TCTTTT	32072	31873	31969	31980	31969	32008	rpl2
CCCCCT	32085	31886	31982	31993	31982	32021	rpl2
CAACAA	32114	31915	32011	32022	32011	32050	rpl2
AAAACA	32159	31960	32056	32067	32056	32095	rpl2
CCCCCA	32180	31981	32077	32088	32077	32116	rpl2
TTTTTC	32204	32005	32101	32112	32101	32140	rpl2
CCCCCT	32267	32068	32164	32175	32164	32203	rpl2
TCTTTT	32321	32122	32218	32229	32218	32257	rpl2
GGGGAG	32396	32197	32293	32304	32293	32332	rpl2
AAAAGA	32621	32422	32518	32529	32518	32557	rps19
CCTCCC	32651	32452	32548	32559	32548	32587	rps19
TTTTGT	32768	32569	32665	32676	32665	32704	rps3
TTTTTC	32774	32575	32671	32682	32671	32710	rps3
GGGGCG	32823	32624	32720	32731	32720	32759	rps3
TTTTCT	32978	32779	32875	32886	32875	32914	rps3
TTTTTC	32981	32782	32878	32889	32878	32917	rps3

Table S8.2. Continued

ACAAAA	32986	32787	32883	32894	32883	32922	rps3
CCTCCC	32997	32798	32894	32905	32894	32933	rps3
CTTCTT	33020	32821	32917	32926	32917	32956	rps3
CCCCAC	33071	32872	32968	32979	32968	33007	rps3
TTTTTA	33083	32884	32980	32991	32980	33019	rps3
GGGGGA	33085	32886	32982	32993	32982	33021	rps3
CCACCC	33257	33058	33154	33165	33154	33193	rps3
CCTCCC	33276	33077	33173	33184	33173	33212	tps3
ATTATT	33325	33126	33222	33233	33222	33261	rps3
AAAAAG	33359	33160	33256	33267	33256	33295	rps3
CAACAA	33362	33163	33259	33270	33359	33298	rps3
GGGGGA	33605	33406	33502	33513	33502	33541	rpl16
GAAGAA	33682	33483	33579	33590	33579	33618	rpl16
GAAGAA	33770	33501	33597	33608	33597	33636	rpl16
GGGGAG	33709	33510	33606	33617	33606	33645	rpl16
CCCCCT	33714	33515	33611	33622	33611	33650	rpl16
TTTTTC	33721	33522	33618	33629	33618	33657	rpl16
GAAGAA	33754	33555	33651	33662	33651	33690	rpl16
TTTTTC	33766	33567	33663	33674	33663	33702	rpl16
GGGGGA	33779	33580	33676	33687	33676	33715	rpl16
CCCCCT	33799	33600	33696	33707	33696	33735	rpl16
CTTCTT	33806	33607	33703	33714	33703	33742	rpl16
AAAAAG	33907	33708	33804	33815	33804	33843	trnI
GGAGGG	33950	33751	33847	33758	33847	33886	trnI
AACAAA	34004	33805	33901	33927	33901	33940	orf217
CCCCAC	34019	33820	33916	33942	33916	33955	orf217
AAAAAG	34173	33974	34070	34096	34070	34109	orf217
CTTCTT	34189	33990	34086	34112	34086	34125	orf217
AAAACA	34263	34064	34160	34186	34160	34199	orf217
CCCCCT	34312	34113	34209	34235	34209	34248	orf217
GGGGGT	34330	34131	34227	34253	34227	34266	orf217
AGAAAA	34331	34132	34228	34254	34228	34267	orf217
CCCCCT	34381	34182	34278	34304	34278	34317	orf217
GGGGGA	34390	34191	34287	34313	34287	34326	orf217
CCTCCC	34410	34211	34307	34333	34307	34346	orf217
CCTCCC	34418	34219	34315	34341	34315	34354	orf217
GGAGGT	34421	34222	34318	34344	34318	34357	orf217
ATTATT	34457	34258	34354	34380	34354	34393	orf217
ATTATT	34458	34259	34355	34831	34355	34394	orf217
AAAAGA	34539	34340	34436	34462	34436	34475	orf217
CTCCCC	34543	34344	34440	34466	34440	34479	orf217
AAAACA	34572	34373	34469	34495	34469	34508	orf217
AAAACA	34643	34444	34540	34566	34540	34579	orf217
TTTCTT	34736	34537	34633	34644	34633	34672	atp8

Table S8.2. Continued

AAAAAC	34757	34558	34654	34665	34654	34693	atp8
AAAAAG	34832	34633	34729	34730	34729	34768	atp8
ATAAAA	34864	34665	34761	34772	34761	34800	atp8
GGAGGG	34950	34751	34847	34858	34847	34886	atp8
CCCCCT	34975	34776	34872	34883	34872	34911	atp8
TCCTCT	35007	34808	34904	34915	34904	34943	atp8
CCTCAC	35029	34830	34926	34937	34926	34965	atp8
TTTTAT	35030	34831	34927	34938	34927	34966	atp8
TTTTGT	35031	34832	34928	34839	34928	34967	atp8
AAAAAT	35046	34847	34943	34954	34943	34982	atp8
TTTTTA	35047	34848	34944	34955	34944	34983	atp8
GGGGGA	35053	34854	34950	34961	34950	34989	atp8
AAAAAG	35065	34866	34962	34973	34962	35001	atp8
AAAAGA	35342	35144	35240	35250	35240	35279	rps14
GGGGAG	35404	35206	35302	35312	35302	35341	rps14
AAAAGA	35418	35220	35316	35326	35316	35355	rps14
CTCCCC	35436	35238	35334	35344	35334	35373	rps14
GGAGGG	35506	35308	35404	35414	35404	35443	rps14
AGGAGG	35529	35331	35427	35437	35427	35466	rps14
CCTCTT	35535	35337	35433	35444	35433	35472	rps14
GGGGGA	35551	35353	35449	35459	35449	35488	rps14
TTTTTA	35562	35364	35460	35470	35460	35499	rps14
CCTCCT	35556	35458	35556	35564	35554	35593	rps8
CCTCCC	35662	35464	35562	35570	35560	35599	rps8
CCCCTC	35692	35494	35592	35600	35590	35629	rps8
AAGAAA	35728	35530	35628	35636	35626	35665	rps8
TTCTTT	35785	35587	35685	35693	35683	35722	rps8
CTCCCC	35820	35622	35720	35728	35718	35757	rps8
AAAAAC	35918	35720	35818	35826	35816	35855	rps8
TTTTTA	35932	35734	35832	35840	35830	35869	rps8
GGGGGA	35941	35743	35841	35849	35839	35878	rps8
TTGTTT	35962	35764	35862	35870	35860	35899	rps8
AATAAA	36011	35813	35910	35919	35908	35948	rpl6
AAAAAG	36105	35807	36004	36013	36002	36042	rpl6
AAGAAA	36109	35911	36008	36017	36006	36046	rpl6
CAACAA	36127	35929	36026	36035	36024	36064	rpl6
CCCCTC	36136	35938	36035	36044	36033	36073	rpl6
AAAAAC	36137	35939	36036	36045	36034	36074	rpl6
AGGAGG	36237	36039	36136	36145	36134	36174	rpl6
AAAAAG	36255	36057	36154	36163	36152	36192	rpl6
TTATTT	36287	36089	36186	36195	36184	36224	rpl6
AAACAA	36305	36107	36204	36213	36202	36242	rpl6
GGAGGA	36309	36111	36208	36217	36206	36246	rpl6
CCCCCT	36327	36129	36226	36235	36224	36264	rpl6

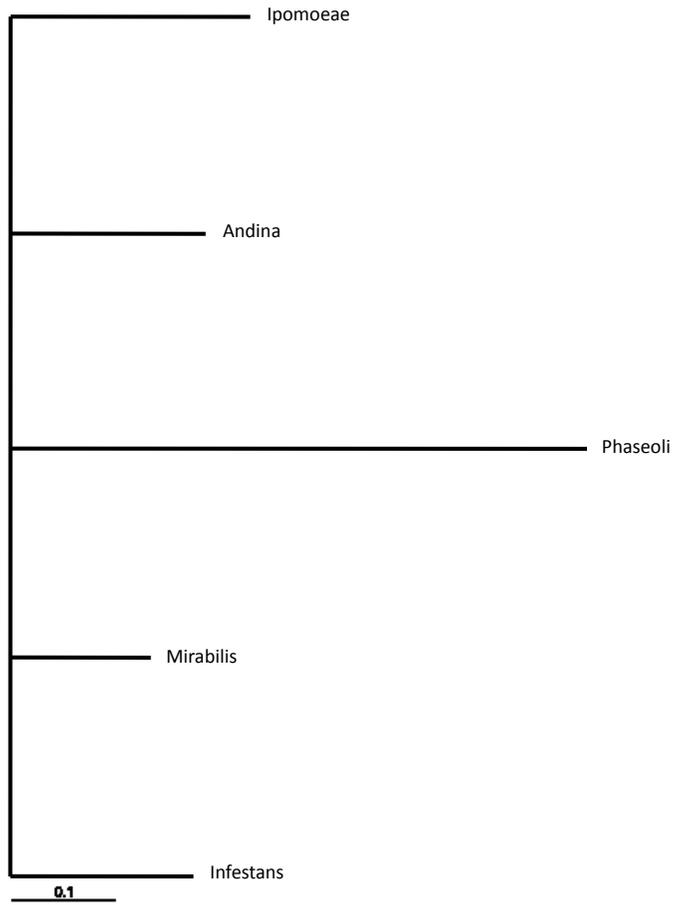
Table S8.2. Continued

AGAAGA	36335	36137	36234	36243	36232	37272	rpl6
TTTTTC	36384	36186	36283	36292	36281	36321	rpl6
TTCTTT	36395	36197	36294	36303	36292	36332	rpl6
ACAAAA	36399	36201	36298	36307	36296	36336	rpl6
AATAAA	36407	36209	36306	36015	36304	36344	rpl6
CTTCTT	36419	36221	36318	36327	36316	36356	rpl6
CTCCCC	36445	36247	36344	36353	36342	36382	rpl6
AAAAGA	36542	36344	36441	36450	36439	36479	rpl6
AAAAGA	36563	36365	36462	36471	36460	36500	rpl6
AAAAAC	36615	36417	36514	36523	36512	36552	rps2
AAAAAG	36649	36451	36548	36557	36546	36586	rps2
AAAAAG	36680	36482	36579	36588	36577	36617	rps2
GAAGAA	36689	36491	36588	36597	36586	36626	rps2
AAAAAC	36716	36518	36615	36624	36613	36653	rps2
AAGAGA	36734	36536	36633	36642	36631	36671	rps2
GGGGGA	36738	36540	36637	36646	36635	36675	rps2
ACAAAA	36816	36618	36715	36724	36713	36753	rps2
AAAAGA	36890	36692	36789	36798	36787	36827	rps2
GTTGTT	36952	36754	36851	36860	36849	36889	rps2
AAAAAG	37025	36827	36924	36933	36922	36962	rps2
AGAAAA	37058	36860	36957	36966	36955	36995	rps2
CTCCCT	37081	36883	36980	36989	36978	37018	rps2
TTGTTT	37133	36935	37032	37041	37030	37070	rps2
TGTTTT	37169	36971	37068	37077	37066	37106	rps2
AACAAA	37184	36986	37083	37092	37081	37121	rps2
GGGGGA	37327	37129	37226	37235	37224	37264	rps4
GGGGGA	37333	37135	37232	37241	37230	37270	rps4
AAAACA	37484	37286	37383	37392	37381	37421	rps4
TTTTGT	37517	37319	37416	37425	37414	37454	rps4
AGAAAA	37588	37390	37487	37496	37485	37525	rps4
AAAATA	37595	37397	37494	37503	37492	37532	rps4
TTGTTT	37684	37486	37583	37592	37581	37621	orf100
AAAAAT	37725	37527	37624	37633	37622	37662	orf100
CTTCTT	37727	37529	37626	37635	37624	37664	orf100
ACAAAA	37800	37602	37699	37708	37697	37737	orf100
TTTTTA	37875	37677	37774	37783	37772	37812	orf100
GAAGAA	37879	37681	37778	37787	37776	37816	orf100
CCACCC	37881	37683	37780	37789	37778	37818	orf100
TTCTTT	37891	37693	37790	37799	37788	37828	orf100
TCTTTT	37924	37726	37823	37832	37821	37861	orf100
AAAGAA	37972	37774	37871	37880	37869	37909	orf100

^a Polymorphism corresponds to the position of the nucleotide in *P. infestans*, *P. phaseoli*, *P. andina* (Ic), *P. andina* (Ia), *P. ipomoeae*, and *P. mirabilis* respectively.

APPENDIX C

Figure S8.1. Phylogenetic tree of *Phytophthora* species in Ic clade constructed using MrBayes. Collapsed hapmap sequences were used to run the analysis.

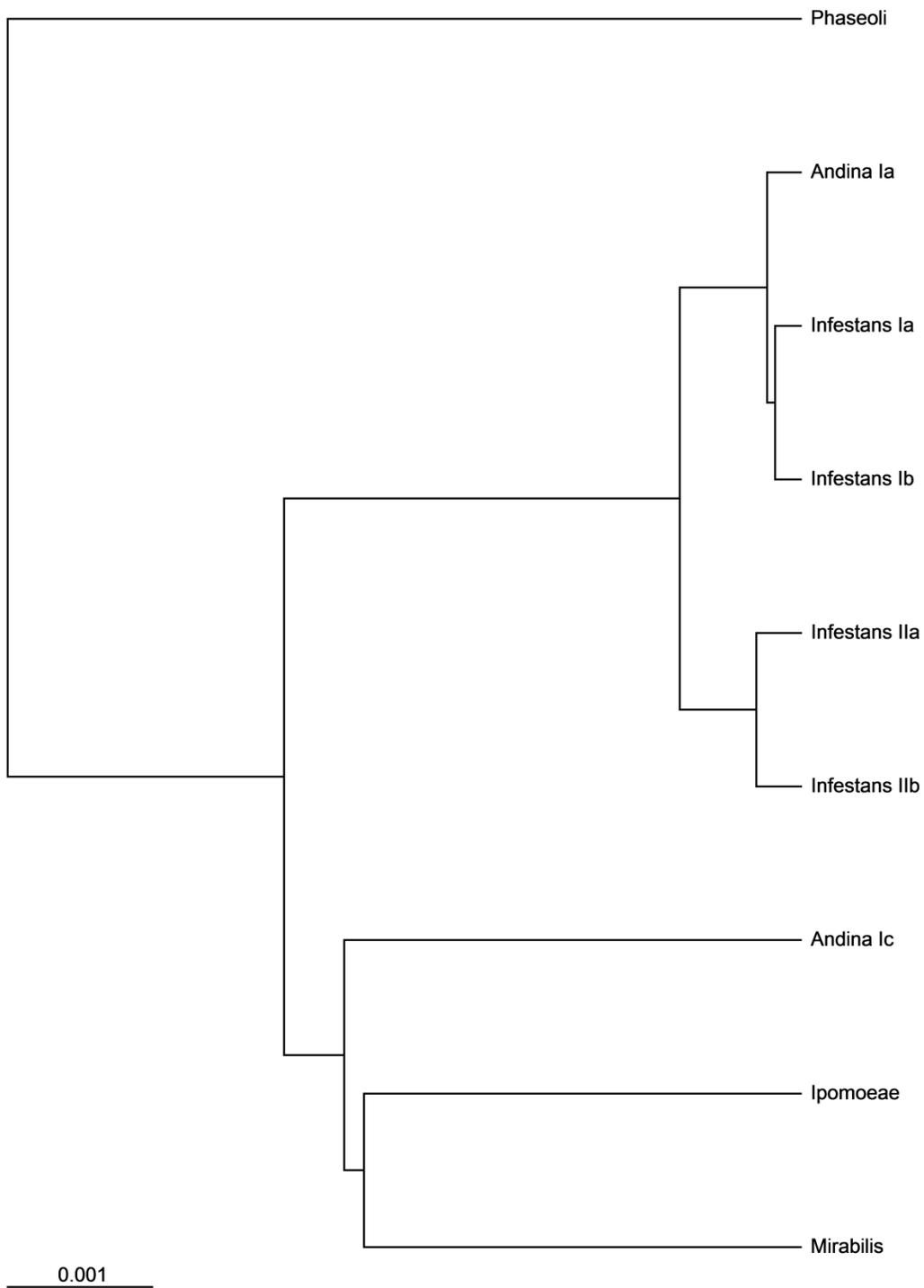


APPENDIX D

Figure S8.2. Collapsed haplotypes of *Phytophthora* Ic clade species used for coalescent analysis, including transitions (t) and transversions (v). Numbers at the top indicate the numbered mutation. Asterisks indicate sites that are incompatible with variation that is fully compatible in the largest nonrecombining partition.

APPENDIX E

Figure S8.3. Evolutionary analysis of *Phytophthora* Ic clade species mitochondrial genomes using BEAST. *P. phaseoli* diverged earliest from all other species, while *P. ipomoeae* and *P. mirabilis* diverged most recently.



CHAPTER 9

CONCLUSIONS AND FUTURE RESEARCH – NON-MENDELIAN INHERITANCE OF
GENOMIC AND EXTRA-GENOMIC INFORMATION, AND OTHER TOPICS THAT COULD
COFUUSE THE CLASSICAL GENETICIST

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Introduction

In 1866, Gregor Mendel, in his paper *Versuche über Pflanzenhybriden* (*Experiments on Plant Hybrids*) described the laws that would later become the basis for the field of Genetics. The First Law (a.k.a., Law of Segregation) states that gamete producing individuals pass on only one of two alleles of any particular gene to each offspring (Mendel, 1951). The Second Law (a.k.a., Law of Independent Assortment) states that alleles of genes [not including those that are tightly linked] assort independently of one another during gamete formation (Mendel, 1951). At first his theories weren't accepted and were greatly criticized. Eventually his ideas would be swept under the rug and become forgotten. In 1900, Mendel's theories were re-discovered and ever since have been widely accepted; however, since then have continued to face scrutiny and opposition. In this review I aim to describe a few concepts that deviate from Mendel's laws; from mechanisms that violate the two laws thereby resulting in hereditary estimates deviating from the typical inheritance ratios (e.g., extranuclear/cytoplasmic and uniparental inheritance) to other mechanisms that consider either the gain or loss of genetic information (e.g., aneuploidy, gene conversion, copy number variation; and conditionally supernumerary/dispensable/B/minichromosomes), and additional mechanisms in which multiple nuclei (e.g., micronuclei, nuclear migration and Buller's phenomenon) or extra genomic information are involved. The point of this article is not to pinpoint every single way in which Mendel's laws may be violated, but is to describe a few of the major ways and give examples of each. In the second part of this review, I will offer suggestions to the biological significance of such concepts and then propose

an idea that incorporates a few of these topics to help answer a question related to my work on *Aspergillus flavus*.

Extranuclear/cytoplasmic, infectious heredity and uniparental inheritance

In 1902, shortly after the re-emergence of Mendel's laws, it was shown that these hereditary factors were exclusive to chromosomes (Kuroiwa, 2010a); then in 1909, German botanists Erwin Bauer and Carl Correns, both, independently showed that some genes in *Pelargonium* (geranium) and *Mirabilis jalapa*, respectively, violate Mendel's laws. They showed that the leaf color trait was exclusively maternally inherited (Hagemann, 2000). For example, Correns showed that green branches always gave rise to green seedlings, and seeds from yellow branches yielded only yellow offspring. In contrast, the pollen-providing (i.e., male) parent had no influence on the phenotype of the progeny (Hagemann, 2000). They concluded that in addition to the Mendelian inheritance of genes in the cell nucleus, there are other hereditary factors outside the nucleus that exhibit a non-Mendelian mode of inheritance. Later research would prove that these non-Mendelian inheritable factors resided within the chloroplast (Sager, Lane, 1972; Sager, Ramanis, 1965) and mitochondria (Giles *et al.*, 1980; Mitchell, Mitchell, 1952). Subsequently, non-Mendelian inheritance was discovered in filamentous fungi, notably *Aspergillus nidulans*, *Neurospora crassa*, and *Podospora anserina* (Kuroiwa, 2010a).

Maternal inheritance is caused not only by physical processes such as differences in gamete volume (e.g., female gametes tend to be much larger than male

gametes), but also by biochemical reactions based on systemic gene expression. The uniparental transmission of plastids may result from the active digestion of the male organelle nuclei post fertilization in young zygotes by nucleases in most plants (Kuroiwa, 2010b). Similar mechanisms degrade mitochondrial genomes from one parent in animals and fungi (Kuroiwa, 2010b). Other modes of maternal inheritance may occur during other phases of development; for example, during gametogenesis where organelles may be segregated from the gamete during pre-meiotic or meiotic divisions; or during fertilization as the paternal organelle DNA may not enter the egg (maternal organelle) (Birky, 2001).

Uniparental inheritance of chloroplasts and mitochondria is not exclusively maternal. Paternal inheritance of chloroplast DNA has been found to be the predominant mode of inheritance in conifers and other gymnosperms, and more and more cases are being established for paternal inheritance of the chloroplast in angiosperms (Yang *et al.*, 2000). For example Yang *et al.* (2000) in interspecific crosses within the genus *Larrea*, examined 20 F1s, and through PCR and RFLP, found in each case, that it was the paternal chloroplast DNA marker that appeared in the F1 individuals (Yang *et al.*, 2000). Length polymorphism was the basis for differentiating the 3 species used, and matings were done in a reciprocal manner to ensure that preferential transmission by species was not occurring (Yang *et al.*, 2000). There are several ways in which maternal plastids may not be inherited by the offspring. First, plastids may be absent from the initial stage of egg cell development by unequal distribution of plastids during the formation of megaspores (Hagemann, 2002). Second,

plastids may be present in the young egg cell but may be greatly deformed or degenerated during egg cell development, such as in the conifers (Yang *et al.*, 2000). Third, if plastids are present, they may be positioned in or moved to the regions of the cell at fertilization that will not become a part of the functional embryo (Mogensen, 1996). Fourth, if maternal plastids do become part of the functional embryo, they may still be eliminated during early development of the offspring; as is seen during embryogenesis in alfalfa (Rusche *et al.*, 1995). The same phenomena of paternal inheritance with very similar mechanisms was also found to occur in mammals (Schwartz, Vissing, 2002), algae (Nakamura, 2010) and fungi (Yang, Griffiths, 1993).

Aneuploidy, gene conversion and copy number variation

This next section will be devoted to the ways in which a gain or loss of whole chromosomes (aneuploidy) or segments of chromosomes (segmental aneuploidy, gene conversion and copy number variation) may occur, which, if the resultant organism is viable and fertile, may vertically transmit said chromosomes to their progeny.

Aneuploidy is defined as a chromosome number that is not an exact multiple for the haploid number. This condition is distinct from the condition of polyploidy, which is defined as having a chromosome number that is a multiple greater than two of the monoploid number (Torres *et al.*, 2008). Polyploidy is frequently found in nature and can be part of the normal physiology of plants and animals; on the other hand, aneuploidy frequently causes lethality and has been associated with disease, sterility and tumor formation (Torres *et al.*, 2008).

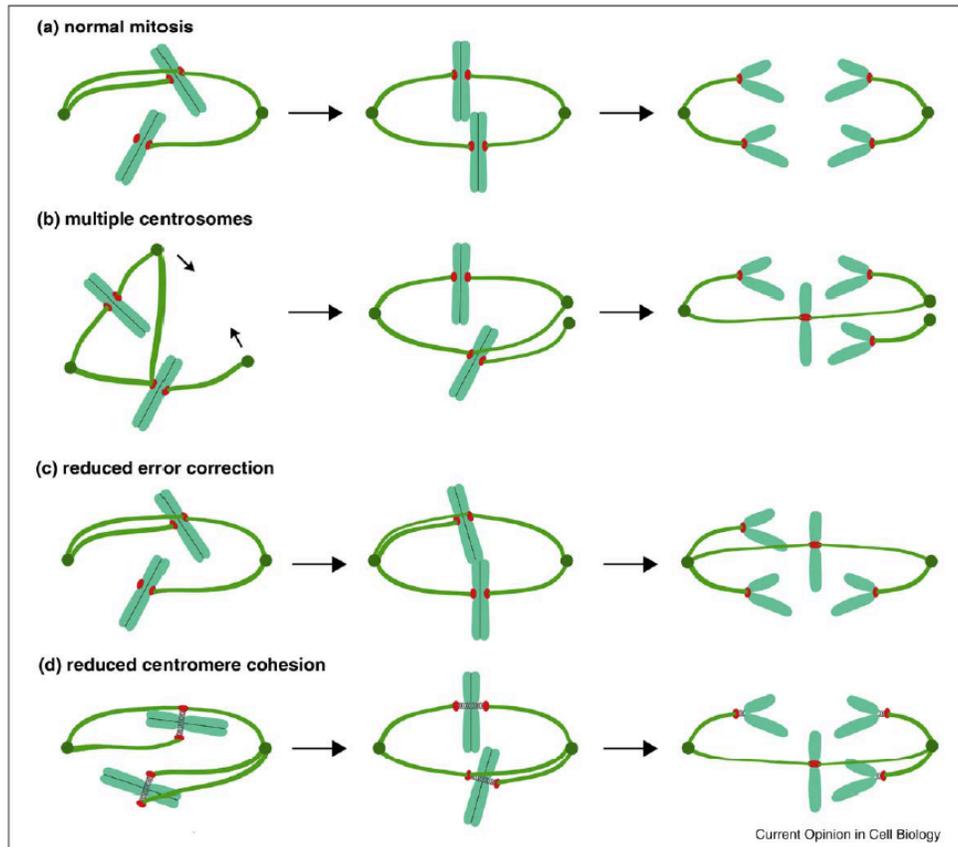
The first systematic analysis of the effects of aneuploidy on cell and organismal physiology was performed more than a century ago in sea urchins by Theodor Boveri. He examined the development of sea urchin eggs that were fertilized by two sperm and hence were triploid and therefore formed four, rather than two, centrosomes during the first embryonic mitosis. This led to the formation of a tetrapolar spindle during the first mitosis; chromosomes segregated to the four poles, generating a four-celled embryo skipping the two-celled stage. The result of this division was massive aneuploidy (Torres *et al.*, 2008). Boveri observed that the embryos that resulted from dispermic fertilizations exhibited developmental defects and died. Only those doubled-fertilized embryos that by chance received the species-typical chromosomal complement developed into normal larvae; protein stoichiometry imbalances leading to developmental defects will be discussed later. Boveri concluded that chromosome gain or loss leads to abnormal development and lethality (Torres *et al.*, 2008).

Reports of aneuploidy in the fruit fly *Drosophila melanogaster* were published in the early 1920s when Calvin Bridges showed that a *Drosophila* mutant known as 'Diminished' was monosomic for the smallest chromosome. These flies were smaller in size, sterile and exhibited many developmental abnormalities (Bridges, 1921a; Bridges, 1921b). Later research in the 1970s by Lindsley *et al.* (1972), in which they created thousands of *Drosophila* mutant lines that either carried additional fragments of different chromosomes (segmental trisomies) or lacked different chromosomal regions (segmental monosomies) by crossing flies containing translocations between the Y chromosomes and autosomes, showed that: 1) segmental monosomies are less well

tolerated than segmental trisomies, 2) extensive hyperploidy (i.e., large segments of genome being trisomic) is lethal, and 3) intermediate hyperploidy results in a set of traits that is independent of the identity of the triploid segment. Together, these results showed that aneuploidy causes a series of defects that become more pronounced as the size of the trisomic segment increases (Lindsley *et al.*, 1972).

Various events contribute to chromosome mis-segregation that leads to aneuploidy, including: defects in spindle geometry, merotelic kinetochore-microtubule dynamics, and chromosome cohesion (Compton, 2011) (see Figure 9.1). Most of these mechanisms converge on a common pathway to cause chromosome mis-segregation by undermining the appropriate attachment of kinetochores to spindle microtubules (Compton, 2011). During cell division, spindle microtubules attach to chromosomes through specialized structures called kinetochores that assemble on each sister chromatid adjacent to the centromere. The separation of sister kinetochores by centromeric chromatin ensures their back-to-back geometry, and this geometric constraint ensures segregation accuracy by promoting attachment of each kinetochore to microtubules oriented toward only one spindle pole. The timing of sister chromatid separation at anaphase is regulated by a checkpoint, which signals a pathway that monitors the attachment of spindle microtubules to kinetochores (Compton, 2011). Breaching of this checkpoint may result in aneuploid daughter cells.

Thompson and Compton (2008) showed that the most common cause of chromosome mis-segregation in aneuploid cells is lagging chromosomes at anaphase (Thompson, Compton, 2008). Kinetochores on the lagging chromosomes have



Mechanisms for chromosome mis-segregation during cell division. Many mechanisms causing chromosome mis-segregation arise from defects in the attachments of spindle microtubules to kinetochores. **(a)** Errors in kinetochore–microtubule attachments occur spontaneously in early phases of mitosis but are efficiently corrected in normal cells to promote accurate chromosome segregation. **(b)** Extra centrosomes increase the frequency of kinetochore–microtubule attachment errors by inducing transient defects in spindle geometry. **(c)** Correction of erroneous kinetochore–microtubule attachments relies on the release of improperly attached microtubules and hyperstable kinetochore–microtubule attachments reduces the efficiency of correction. **(d)** Defects in centromere cohesion disrupt the back-to-back geometric constraints on sister kinetochores, which increases the formation rate of improper kinetochore–microtubule attachments.

Figure 9.1. (from [Compton, 2011]): Mechanisms for chromosome mis-segregation

microtubules oriented toward both spindle poles in an arrangement called merotelly (Cimini *et al.*, 2001). Merotelic kinetochores achieve appropriate numbers of microtubule attachments and they effectively satisfy the spindle assembly checkpoint and do not present anaphase onset (Cimini *et al.*, 2001). The correction of merotelic kinetochore microtubule attachments relies on the release of improperly attached microtubules, however direct analyses have shown that kinetochore microtubule attachments in aneuploid tumor cells are stable, compromising their ability to correct erroneous kinetochore microtubule attachments.

Bridging the concepts from the previous section on uniparental inheritance and the concepts of this section of aneuploidy, is the concept of inheritance of more than two alleles at a locus and noninheritance of any alleles from either parent at a locus. This phenomenon seems to be common in the *Phytophthoras* as research groups have found evidence of this in *P. nicotianae* (Forster, Coffey, 1990), *P. infestans* (Carter *et al.*, 1999), *P. cinnamomi* (Dobrowolski *et al.*, 2002), and most recently in *P. ramorum* (Vercauteren *et al.*, 2011). In the study that involved *P. cinnamomi*, the researchers developed microsatellite markers to examine their inheritance in 200 sexual progeny of four separate crosses (Dobrowolski *et al.*, 2002). Results indicated that a large proportion of the sexual progeny from all four crosses showed non-Mendelian inheritance of alleles. This included the inheritance of alleles from only one parent at a locus in an otherwise outcrossed progeny. For example, in cross 1, locus *d39*, six progeny inherited only one allele from the A1 parent, and no allele from the A2 parent, whereas two offspring inherited only the *136* allele from the A2 parent. A1 and A2 refer

to the two different mating types. Also, more than two alleles at a locus were inherited, e.g., nine progeny in cross 1, locus *g10*, inherited three different alleles, with at least two coming from one parent (Dobrowolski *et al.*, 2002). A high proportion of imperfect meiosis in the parents could explain this non-Mendelian inheritance. Nondisjunction at the first division of meiosis would give gametic nuclei fewer or more chromosomes than expected from strict segregation. This would result in aneuploid progeny, which would explain the patterns of inheritance seen among the microsatellite loci. Other than nondisjunction, heterokaryosis is a hypothesis that may explain these results. Heterokaryotic progeny would arise if more than one fused nucleus survived in the oospore and then divided during germination.

The incidence of aneuploidy increases dramatically as women age indicating that there is an age-associated deterioration in oocyte quality. The loss of cohesion correlated with reduced quantities of the cohesion subunit Rec8 associated with chromosomes, and fits with data from other model systems showing that reduced cohesion can increase mis-segregation rates in oocytes (Subramanian, Bickel, 2008). The reduction of centromeric cohesion disrupts the normal geometric constraint that favors the attachment of kinetochores to spindle microtubules from opposing spindle poles. Thus, oocytes from older mice have a greatly enhanced frequency with which the kinetochores attach to spindle microtubules oriented toward the wrong spindle pole and this elevates chromosome mis-segregation rates. This attenuates the back-to-back geometric constraint on sister kinetochores and greatly increases the frequency of

merotelic attachments that elevates chromosome mis-segregation rates leading to aneuploidy.

Gene conversion is an event during genetic recombination, in which DNA sequence information is transferred from one sister chromatid to another sister chromatid. As a consequence, the 'acceptor' sequence is replaced, wholly or partly, by a sequence that is copied from the 'donor', whereas the sequence of the donor remains unaltered. In eukaryotes, gene conversion constitutes the main form of homologous recombination that is initiated by DNA double-strand breaks. Gene conversion mediates the transfer of genetic information from intact homologous sequences to the region that contains the double-strand break, and it can occur between sister chromatids, homologous chromosomes or homologous sequences on either the same chromatid or different chromosomes (Chen *et al.*, 2007). According to the double-strand break repair model (see Figure 9.2), the ends of the double strand break are cut by 5' → 3' exonucleases, resulting in the formation of two 3' ssDNA tails. These tails actively 'scan' the genome for homologous sequences; one of them invades the homologous DNA duplex to form a displacement (D)-loop, which is then extended by DNA synthesis, having been primed from this single-end invasion. The extended D-loop then pairs with the other 3' ssDNA tail (second-end capture), while DNA synthesis at the newly captured strand followed by ligation of the nicks results in the formation of an intermediate with two Holliday junctions (HJs). An HJ is a point at which the strands of two dsDNA molecules exchange partners, an event that occurs as an intermediate in crossing over or gene conversion. Random cleavage of the two HJs by an HJ resolvase

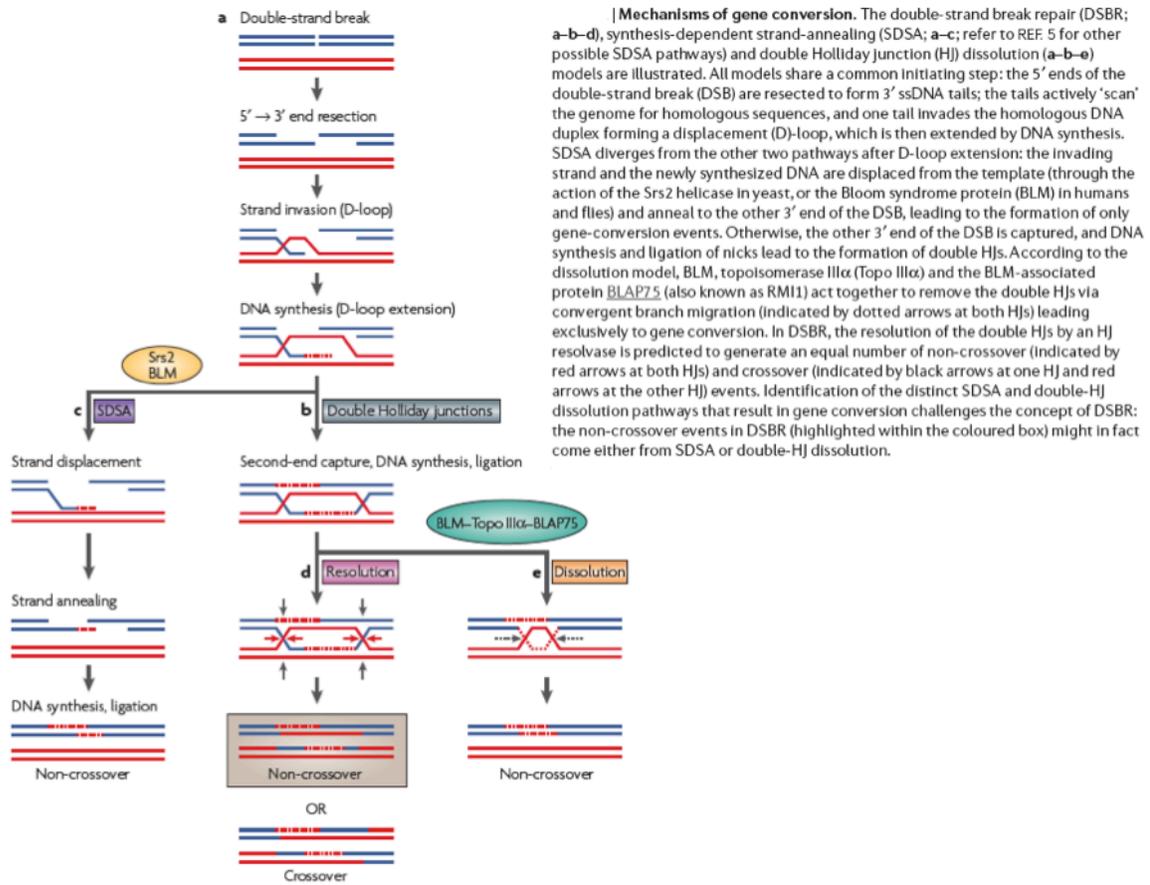


Figure 9.2 (from (Chen *et al.*, 2007)): Mechanisms of gene conversion

yields either a non-crossover (i.e., gene conversion) or a crossover product. During this process, most gene conversion is derived from mismatch repair of the heteroduplex DNA that is formed between the donor and acceptor DNA sequences (Szostak *et al.*, 1983). Another model that results in only non-crossover products is the synthesis-dependent strand-annealing model (SDSA) (see Figure 9.2), which after strand invasion and D-loop extension, the newly synthesized strand is displaced from the template and anneals to the other 3' ssDNA tail, and followed by DNA synthesis and ligation of nicks (Szostak *et al.*, 1983). Another mechanism, known as double-HJ dissolution (see Figure 9.2), shares the two defining characteristic features of the synthesis-dependent strand-annealing model in terms of outcome; however, it generates its non-crossover product from the convergent migration of the two HJs towards each other, leading to the collapse of the double HJs (Szostak *et al.*, 1983).

Copy-number variation (CNV) is a form of structural variation that is an alteration of the DNA of a genome resulting in the cell having an abnormal number of copies of one or more sections of DNA. CNVs correspond to relatively large regions of the genome that have been deleted or duplicated. CNVs contrast with single-nucleotide polymorphisms, which affect only a single nucleotide base. CNVs can be caused by structural rearrangements of the genome such as deletions, duplications, inversions and translocations (Lee, Lupski, 2006). The architectural features that appear to render genomic regions susceptible to rearrangement include region-specific repeat sequences, or low-copy repeats (LCRs). LCRs are sequences that occur twice or a few times in a haploid genome (Hastings *et al.*, 2009). LCRs can contain one or multiple

genes, pseudogenes, gene fragment, retroviral sequences, regulatory regions, or other paralogous segments and are characterized as repeat sequences, as opposed to highly repetitive sequences (e.g., satellite DNA) (Lee, Lupski, 2006). LCRs are often found in pericentromeric and subtelomeric regions of the chromosomes, but they may be found in interstitial regions as well. The size, relative orientation, distance between copies, and shared percent identities of the LCRs are factors that render the genomic region susceptible to rearrangement and may influence the type of rearrangement that may occur (Lee, Lupski, 2006).

As afore mentioned, gains or losses of chromosomes or pieces of chromosomes usually lead to disorders and defects. Imbalances in protein stoichiometry are likely to be the cause of defects in organisms with extra chromosomes. In the case of chromosomes losses, two reasons are likely to be responsible for the defects: 1) a reduction in protein activity due to the reduction in gene dosage, which is known as haplo-insufficiency, and 2) as in the case of chromosome gains, protein stoichiometry imbalances (Torres *et al.*, 2008). An example that shows losses leading to a reduction in gene dosage is in *Drosophila* in which a loss of one copy of 64 out of a total of 79 cytoplasmic ribosomal proteins leads to the 'Minute' phenotype, with flies being small and exhibiting poor fertility and viability (Marygold *et al.*, 2007). On the other side of that notion, an example of chromosome gains leading to an imbalance of protein stoichiometry is with the gene encoding β -tubulin. In budding yeast, the presence of an extra copy of the β -tubulin-encoding genes in cells is lethal, but cells survive only when they harbor an additional copy of chromosome 13, on which the α -tubulin genes are

located (Katz *et al.*, 1990). Torres *et al.* (2007) showed that haploid and diploid yeast strains containing an extra chromosome show a corresponding increase in transcript levels; diploid yeast strains lacking a chromosome show a matching decrease (Torres *et al.*, 2007). The balance theory explains that aneuploidy results in deviations from the normal stoichiometry of protein complex subunits and it is these changes in intracellular protein composition that causes defects in many cellular processes, ultimately leading to developmental defects and a decrease in organismal fitness (Torres *et al.*, 2008).

Supernumerary, B, conditionally dispensable and mini chromosomes

Within a species, a subset of individuals may have more than the minimal complement of chromosomes. If the extra chromosomes are composed primarily of DNA not found in all representatives of the species, they are most appropriately referred to as supernumerary chromosomes (Covert, 1998). 'Supernumerary chromosome' has been used to refer to one of the characteristic attributes of B chromosomes. B chromosomes are recognized as chromosomes that 1) are dispensable and can be present or absent from individual within a population; 2) they do not pair or recombine with any member of the standard diploid (or polyploid) set of A chromosomes at meiosis; and 3) their inheritance is non-Mendelian and irregular (Jones, Houben, 2003).

The significance of B chromosomes is to be found in their widespread occurrence in hundreds of flowering plants, gymnosperms, ferns, byrophytes, fungi, and even in animals, including mammals (Jones, Houben, 2003). The emerging view in

plants is that the B chromosomes are parasitic elements that maintain their polymorphism by drive and that there is a host-parasite relationship between the A and B chromosomes (Jones, Houben, 2003); otherwise, the genetic significance of B chromosomes, as well as their origin, adaptive properties and evolutionary purpose remain unclear (Bougourd, Jones, 1997). With reference to B chromosomes, the term drive refers to the occurrence when the chromosome number in the gametes is greater than Mendelian expectations (i.e., >0.5) (Jones, Houben, 2003). In some cases, this can occur at meiosis, when the B chromosomes migrate to one pole of the spindle during the first anaphase and then pass preferentially into the nucleus that is destined to form the egg cell (Jones, Houben, 2003). Alternatively, drive can occur in the first pollen grain mitosis, when the B-chromatids fail to separate and both pass into the generative nucleus (Jones, Houben, 2003). The inheritance of B chromosomes is non-Mendelian and irregular owing to degrees of meiotic elimination and to various drive processes.

In general, the effects of B chromosomes are usually either neutral or deleterious in nature; in rye, they have been interpreted as purely selfish chromosomes with no advantageous properties, however in chives they are seen as adaptive and beneficial (Bougourd, Jones, 1997) (see section entitled 'models for biological importance'). The extra DNA contributed by the B chromosomes in rye is without genes; they are composed of repetitive sequences that are mostly common with those of the A chromosomes, such that in genomic *in situ* hybridization using whole genome DNA of the A chromosomes, both the B chromosomes and A chromosomes are covered by the signal (Bougourd, Jones, 1997). B chromosomes in rye cause a reduction in

physiological activity from the level of the nucleus and the cell through the whole plant. These negative effects are quantitative and proportional, such that a plant with 10 B chromosomes, found only under experimental conditions, makes at most 3-4 cm of growth and then dies (Bougourd, Jones, 1997). Plants with 2 B chromosomes show little or no change, but as the numbers increase the effects are progressively debilitating (Bougourd, Jones, 1997). Contrary to the previous point, a plant possessing an odd number amount of B chromosomes seem to have a stronger negative effect compared to one that contains an even number; so plants with 1 B chromosome are more adversely affected than those with 2, and those with 3 are more so adversely affected than those with four (Bougourd, Jones, 1997).

Supernumerary chromosomes that carry adaptive traits, such as the supernumerary chromosomes of *Nectria haematococca* mating population VI that carry genes that contribute to the disease-causing capacity of the pathogen on specific host plants, are more accurately referred to as 'conditionally dispensible' chromosomes in order to reflect their importance in some, but not all, growth conditions (Covert, 1998). The 1.6 Mb chromosome of *N. haematococca* was originally called a B chromosome because it was not needed for axenic or saprophytic growth. However, this chromosome and other supernumerary chromosomes from *N. haematococca* mating population VI have been shown to contain specific genes that allow the host isolate to grow in additional biological niches (Taga *et al.*, 1999).

Johnson *et al.* (2001) showed that a conditionally dispensible chromosome of 1.1 Mb from an isolate of *Alternaria alternata* apple pathotype (O-210) appeared to be non-

essential for normal growth but necessary for pathogenicity (Johnson *et al.*, 2001). The apple pathotype of *A. alternata* produces the host-specific toxin AM-toxin, and causes Alternaria blotch on a narrow range of susceptible apple cultivars (Kohmoto *et al.*, 1977). Johnson *et al.* cloned a cyclic peptide synthetase gene, *AMT*, whose product catalyzes the production of AM-toxin (Johnson *et al.*, 2000) and they also showed that it resides on a small chromosome of 1.1 Mb in the *A. alternata* apple pathotype (Orbach *et al.*, 1996). Their laboratory sub-cultured strain O-210 Δ C had been sub-cultured at least ten times in a minimum period of 12 months and was shown to have lost the 1.1 Mb chromosome that contained the *AMT* gene. They showed this through contour-clamped homogeneous electric field electrophoresis and Southern hybridization. Chromosome loss coincided with loss of the *AMT* gene and the inability to produce AM-toxin. An original isolate of strain O-210, which had been stored on a slant and had not undergone sub-culture, was recovered and was found to still contain the 1.1 Mb chromosome; this isolate was able to express the *AMT* gene and produce AM-toxin. They concluded that the small 1.1 Mb chromosome is non-essential for normal growth but necessary for pathogenicity (Johnson *et al.*, 2001). How these *A. alternata* isolates acquire or lose pathogenicity in nature is unknown, especially since there is no known sexual stage; but pathotypes of *A. alternata* depend on the production of host specific toxins for specific virulence, and this spontaneous loss of a conditionally dispensable chromosome containing the *AMT* gene may be one mechanism whereby *Alternaria* isolates can lose specific virulence.

The term 'minichromosome' was adopted by the *Saccharomyces cerevisiae* research community to describe circular plasmids found in this yeast; however, in the filamentous fungal literature, this term is typically used to refer to a subset of small, linear chromosomes within an electrophoretic karyotype (Covert, 1998). Orbach et al. (1996) showed that minichromosomes in *Magnaporthe grisea* are inherited in a non-Mendelian manner and suggested that they are nonessential for growth and pathogenicity, but are associated with low levels of sexual fertility (Orbach et al., 1996). They analyzed the chromosomes of a wide variety of *M. grisea* strains using pulsed-field electrophoresis, in combination with Southern hybridization and genetic crosses. They observed minichromosomes ranging in length from 200 to 2000 kb and noticed that infertile field isolates contained many minichromosomes, whereas all female fertile strains lacked them (Orbach et al., 1996). No functional genes have been defined in the *M. grisea* minichromosomes, and it is not clear if minichromosomes are a result or a cause of the low fertility. Chuma et al. (2003) found minichromosomes to also be common in *M. oryzae* strains and showed that meiotic recombination and sister chromatid reassortments in these minichromosomes may lead to size variation in the minichromosomes vertically transmitted to progeny isolates (Chuma et al., 2003).

The readily observed instability of these supernumerary chromosomes does not necessarily mean that they are less stable during sexual reproduction than their essential counterparts. It may be that their loss and/or truncation is detected simply because they are not required for basic growth, while the loss of essential chromosomes is not detected because their absence is lethal. The function of supernumerary

chromosomes in many species is still unclear, however, in cases like the conditionally dispensable chromosomes found in *N. haematococca*, genes found on the supernumerary chromosomes have been found to be essential for pathogenesis and virulence. The emerging paradigm, therefore, is that genes critical for plant-microbe interactions may be on conditionally dispensable pieces of DNA in fungi. In this context, it is interesting to note that many of the known supernumerary chromosomes in fungi have been identified in field isolates of species that cause plant disease. Whether this association is of broad biological significance remains to be determined, but the collective evidence clearly raises the possibility that as more supernumerary chromosomes in fungi are studied intensively, they will be found to make a rich contribution to genetic diversity and adaptability in many fungal species.

Micronuclei, nuclear migration and Buller's phenomenon

Ciliates such as *Paramecium* have an unusual genetic life-style. These single-celled protozoa maintain two separate sets of DNA. The germline set is found in the micronucleus, which serves as the repository of genetic information for each new sexual generation. The somatic genome is housed within the macronucleus, which is the site of all vegetative gene expression and thus determine a cell's phenotype (Chalker, 2005). Different ciliate species contain different numbers of micronuclei.

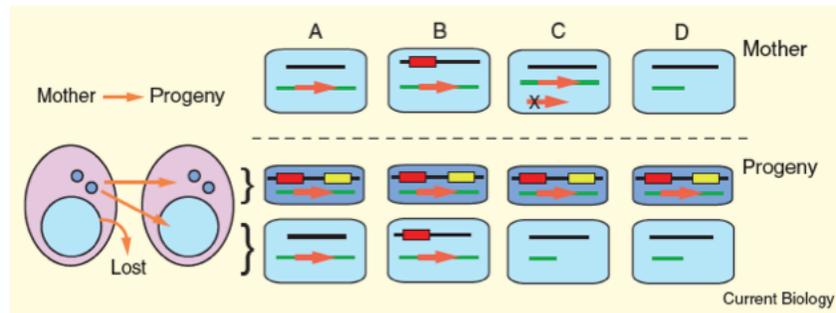
Tetrahymena species have one micronucleus, *Paramecium* species, and *Oxytricha* species usually have two to four. *Stylonychia* species have two micronuclei, *Euplotes* species have one, and, at an extreme, *Urostyla grandis* has between 5 and 20 (Prescott,

1994). The multiple micronuclei in a single organism are all genetically identical; they all are derived by mitosis from one original micronucleus formed by fertilization at cell mating (Prescott, 1994). Micronuclei divide mitotically during vegetative growth, but the form of mitosis is different from that seen in plant and animal cells. Mitosis occurs intranuclearly, i.e., without breakdown of the nuclear envelope, and individual chromosomes are not distinguishable. Rather, the mitotic micronucleus contains long strands of chromatin that distribute to produce two genetically equivalent daughter micronuclei (Prescott, 1994). *Tetrahymena* and *Paramecium* species generally have a single ovoid macronucleus, which is miniscule compared to the hundreds of micronuclei in *U. grandis* (Prescott, 1994). The micronucleus is genetically silent or nearly so during vegetative growth. When a *Paramecium* cell is experimentally constructed to contain alleles that differ in the micronucleus and macronucleus, only the macronuclear alleles govern the cell phenotype (Prescott, 1994). Experimental removal of the micronucleus from various species of ciliates usually results immediately in a lower reproductive rate or renders a cell incapable of vegetative growth, with consequent death (Mikami *et al.*, 1985). This clearly proves that the micronucleus does make a contribution, perhaps expressing a few micronucleus-specific key genes that are absent from the macronucleus and are essential for vegetative growth. On the contrary, amiconucleated ciliates are often found in the wild and proliferate vigorously in laboratory culture. However, the observation that the macronucleus of a viable amiconucleated strain of *T. thermophila* contains a few DNA sequences that are normally restricted to the micronuclear genome and that may represent a particular

gene or genes whose products have a special role in vegetative cell growth (Karrer *et al.*, 1984).

The genomes in the two kinds of nuclei are intimately related as the macronucleus forms from a micronucleus after mating. Therefore, all of the DNA sequences present in the macronucleus are derived from the micronucleus, but the macronuclear sequences are only a subset of the micronuclear sequences (Prescott, 1994). *Tetrahymena* micronuclear and macronuclear DNAs has shown that 10 to 20% of the micronuclear DNA sequence complexity is eliminated in the anlage. Anlage is the term given to the developing macronucleus. Researchers studying the *Tetrahymena* species has identified three global changes in DNA when a micronucleus is converted into a macronucleus: 1) repetitious sequences and some unique sequences (a.k.a., internal eliminated sequences) dispersed throughout the genome are deleted and destroyed; 2) the original five pairs of chromosomal DNA molecules are fragmented in a specific pattern into roughly 200 molecules; and 3) each of the ~200 different subchromosomal molecules is amplified to an average of ~57 copies in the mature macronucleus (Prescott, 1994). Functional significance of these three changes are unclear, however, as a result, the once transcriptionally silenced micronucleus has become the highly active transcriptional macronucleus (Prescott, 1994).

During sexual development the maternal somatic nucleus (macronucleus) is lost and replaced by a new one in the progeny that differentiates from the germline micronucleus. New micronuclei are formed as well (Chalker, 2005) (see Figure 9.3). Meyer *et al.* (2002) have shown that the simple presence or absence of a particular



On the left: two generations of *Paramecium*, showing the fate of the germline and somatic nuclei. During sexual development the maternal somatic nucleus (large, pale blue circle) is lost and replaced by a new one in the progeny that differentiates from the germline micronucleus (small, dark blue circles, two are present in each cell). New micronuclei are formed as well. The normal genetic program (A) can be altered by the pre-existing patterns in the maternal macronucleus via homology-dependent processes (B–D). The content of the mother’s somatic macronucleus is shown above the dashed line and that of the progeny’s micronuclei and macronuclei are shown below. Germline micronuclei of the mother and progeny develop unaltered — only that of the progeny is shown. Two genomic regions are depicted, one containing two internal eliminated sequences (IESs, the red and yellow colored bars) and another containing an expressed gene (the orange arrow with green flanking DNA). The default program (A) is the elimination of the IESs and maintenance of the gene. (B) An IES present in the maternal macronucleus blocks the normally efficient excision of the homologous IES in the developing macronucleus. (C) High-copy, non-expressible transgenes (orange arrow with X) promotes the removal of the homologous sequence either by inducing imprecise internal deletions or chromosome fragmentation. (D) The absence of a locus from the mother’s somatic genome that is normally retained induces the removal of the homologous region from the developing somatic chromosomes.

Figure 9.3 (from (Chalker, 2005)): Maternal DNA rearrangement patterns modify the programming of the progeny’s somatic genome.

sequence within the maternal somatic genome can over-ride the genetic program and epigenetically alter DNA rearrangement patterns (Meyer, Garnier, 2002). For example, internal eliminated sequences that are normally efficiently excised during development can be maintained if the homologous sequence is present in the maternal macronucleus (Duharcourt *et al.*, 1998). Nowacki *et al.* (2005) have discovered putative RNA-binding proteins, encoded by the paralogous *NOWA1* and *NOWA2* genes, that shuttle between the maternal macronuclei and the anlage (Nowacki *et al.*, 2005). Green fluorescent protein fusions showed that the gene products of *NOWA1* and *NOWA2* accumulate into the maternal macronucleus shortly before meiosis of the germline micronuclei and is later transported to the anlage. Consequently, depletion of these proteins impairs the elimination of transposons and of those internal eliminated sequences that are controlled by maternal effects (Nowacki *et al.*, 2005). These results indicate that Nowa proteins are essential components of the *trans*-nuclear-crosstalk mechanism that is responsible for epigenetic programming of genome rearrangements. Characterization of the Nowa proteins showed that the maternal genome can somehow be used to alter development.

As stated by the Buller phenomenon, anastomosis between a homokaryon and a heterokaryon produces a hybrid heterokaryon that receives the nucleus of the homokaryon and one of the two nuclei of the heterokaryon, which must be sexually compatible with the nucleus of the homokaryon (Callac *et al.*, 2006). This process occurs in many basidiomycetes and is generally accompanied by nuclear migration from the heterokaryon through the homokaryon, which becomes heterokaryotic (Callac

et al., 2006). Such migration may enhance the establishment of novel genotypes and genomes, and if only one nucleus of the heterokaryon is sexually compatible with that of the homokaryotic nucleus, no alleles from the incompatible nucleus of the heterokaryon would be vertically transmitted to progeny, therefore creating bias of which alleles from the heterokaryon is carried through successive generations. In a study by Ramsdale and Rayner (1994), they showed that ratios of nuclear genotypes observed in conidia from heterokaryotic strains of *Heterobasidion annosum*, obtained from pairings between sympatrically derived, sib-related and non-sib related homokaryons, commonly deviated from 1:1 or that of classic Mendelian segregation (Ramsdale, Rayner, 1994). Parental homokaryons and sib-related heterokaryons produced conidia with a mean number of nuclei of about two, whereas non-sib-related heterokaryons produced conidia that were predominantly uninucleate. Moreover, whereas conidia containing more than one nucleus germinated most rapidly when derived from homokaryons or sib-related heterokaryons, uninucleate conidia germinated more readily if derived from non-sib-related heterokaryons (Ramsdale, Rayner, 1994). Such differences in distribution of nuclei and germination patterns amongst conidia from heterokaryons, depending on the relatedness of associated nuclear genotypes, may be interpretable in terms of genomic conflict. Genomic conflict is the tendency of unrelated nuclei to segregate individually (Garbelotto *et al.*, 2004). For all combinations, the allocations of nuclei to conidia and their subsequent patterns of germination may be expected to be directed towards the generation of the highest number of 'fit' progeny. The optimal strategy is to produce those types of conidia that

are most vigorous with respect to resource utilization, show the highest germinability, and which also maintain beneficial combinations of genotypes with a high frequency. The production of predominantly uninucleate conidia from associations between conflicting genomes would allow high germinability and promote the escape or loss of a genotype (Ramsdale, Rayner, 1994). Indeed, there appeared to be a strict dominance hierarchy, in which one genotype always outnumbered its partner. The occurrence of asymmetric ratios within an individual mycelium has implications for an increased potential for developmental plasticity, as well as direct effects on the frequency of a genotype in a natural population. Perhaps, in this case, mycelia respond to a range of environmental stimuli by altering their nuclear ratios in an adaptive fashion.

Extra genomic information

This next section will be devoted to cases in which progeny isolates have been found to possess genetic information that was not found in their parents. The best known example comes from Lolle et al. (2005) in which they showed *Arabidopsis* plants homozygous for recessive mutant alleles of the organ fusion gene *HOTHEAD* inherited allele-specific DNA sequence information that was not present in the chromosomal genomes of their parents, but was present in previous generations (Lolle *et al.*, 2005). Homozygous recessive mutant plants (*hth/hth*) were allowed to self-fertilize, and an analysis of their progeny revealed that some of the plants had acquired the wild-type (*HTH*) allele therefore had a wild-type phenotype. These researchers hypothesized that these genetic restoration events were the result of a template-directed process that

makes use of an ancestral RNA-sequence cache (Lolle *et al.*, 2005). Several alternative explanations were suggested; from the trivial explanations, for example: contamination of seed stocks with wild-type seed or out crossing of the *hth* mutant plants with wild-type pollen, to the more technical, such as the involvement of transposons and repeated sequences, or a high rate of random mutation in this particular region or correction of the gene through a gene conversion mechanism (Lolle *et al.*, 2005). All are possible, however further experimentation to prove against these alternative explanations were successful and the authors maintained their stance on the ancestral RNA-sequence cache, primarily because the parents were probed extensively through PCR and DNA blotting to prove that even a cryptic copy of the wild-type allele was not present in the parents.

Since the original publication by Lolle *et al.* (2005), at least four alternative explanations have been suggested. Chaudhury (2005) proposed a mechanism in which the *hth* DNA sequence is restored by a gene conversion mechanism using homologous genomic sequences as templates. In addition, it was suggested that *hth* mutant cuticles may have increased permeability and so the embryo sac could be more porous to DNA fragments arising from the degraded spores, facilitating the homology-based sequence editing (Chaudhury, 2005). Similarly, a model proposed by Ray (2005) involves gene conversion based on chromatin fragments originating from the degenerating non-functional megaspores, which could be taken up by the egg cell and archived in a way that is inaccessible to detection by PCR or DNA hybridization. The supernumerary chromatin fragments could propagate within the meristem and serve as template for

conversion of the altered genome sequences (Ray, 2005)(F3). Comai and Cartwright (2005) proposed a biochemical mechanism of mutagenesis and selection to explain the *hth* reversion phenomena. According to this model, *hth* mutant plants may accumulate a mutagenic and toxic metabolite; the mutagenicity could enhance the rate of reversion, while the toxicity may lead to selection for the *HTH* revertant genotype (Comai, Cartwright, 2005). Finally, Krishnaswamy and Peterson (2006) proposed an alternative mechanism that is based on the phenotypic alteration in *hothead* mutant plants. They proposed that in both *hth/hth* and *hth/HTH* plants, some of the maternal diploid cells may fuse with the developing embryo and become incorporated into the meristematic zone. The number of these 'Latent Legacy Cells' that become incorporated into the developing meristem may be very few, and thus would escape detection by DNA hybridization or PCR of the progeny somatic tissue. However, when the floral meristem forms gametes, the latent legacy cells may pass on their allele to the next generation. Depending on the proportion of latent legacy cells in the floral meristem, the numbers of progeny exhibiting the grandparental genotype may range from 0 to 10% (Krishnaswamy, Peterson, 2007). The major disadvantage of this model is that it proposes two biological phenomena with little or no precedence. First, the fusion of a 'legacy cell' of unknown origin with the developing embryo. Second, the persistence of the 'legacy cell' in a latent state within the meristem from which it can contribute to the gametes giving rise to the following generation. Whatever the real mechanism for the original observations by Lolle et al. remain a mystery.

A second example of extra genomic information being transmitted comes from Nevzgliadova et al. (1998) in which they showed that the *Saccharomyces cerevisiae* yeast strain YPH857 carrying multiple genetic markers was shown to segregate clones that had a differing phenotype designated Ppsu+ (Nevzglyadova *et al.*, 1998). Unlike the original YPH857 culture that carries an unidentified mutation of resistance to cyclohexamide, the Ppsu+ clones exhibited a decreased level of resistance to this inhibitor of protein synthesis. A comparative analysis of total DNA from the YPH857 strain and the Ppsu+ segregants by Southern blotting provided evidence of the presence of an extraneous nucleus in these segregants. Ppsu+ strains were shown to contain wild-type alleles, apart from deletion and insertional alleles typical for the YPH857 strain. They concluded that the 'cryptic' nucleus belongs to a strain of low viability and can survive as an unexpressed DNA in a small fraction of cells (Nevzglyadova *et al.*, 1998). The authors coined the terms 'concealed' and 'cryptic' heterokaryosis and referred to the aberrant nucleus as 'illegitimate' (Nevzglyadova *et al.*, 2001).

Models for biological importance

As afore mentioned, the effect of B chromosomes are either neutral or deleterious in nature; however the following example portrays an instance where B chromosomes actually benefit the organism they are contained within. In chives, B chromosomes have been found to increase germination rates of seeds thereby increasing the proportion of survivors during drought conditions (Bougourd, Jones, 1997). In the natural habitat of the plants located at the River Wye, Powys, UK, the frequency of B-

containing plants reach between 55-65%. The B constitution of a sample of seeds, collected from a B containing population at the River Wye in July, was compared with a sample of seedlings collected from the same area in September of the same year. The seedling sample contained significantly more Bs than the seed sample, and also contained a higher number of Bs per B containing plant. This implied that there was differential selection operating in favor of B containing seedlings during these early crucial stages of the life cycle when levels of mortality are high (Bougourd, Jones, 1997).

The connection between survival and B chromosomes is clear when the natural habitat is evaluated. The chives grow in crevices in rocky outcrops at the sides of the river, and rapid fluctuations occur in the water level even over very short periods of time. During the summer months, when the plants flower profusely and seeds are produced, the rocky crevices are generally high above the level of the water, and drought conditions prevail. However, with a very short period of time, the river level can rise dramatically, washing away any seeds that have not yet germinated together with any unanchored seedlings. Here both rapid germination and the ability to germinate in very small amounts of water are likely to be beneficial, increasing the likelihood that establishment will occur before the river floods (Bougourd, Jones, 1997). The actual mechanism that connects B chromosomes with ability to germinate faster remains a mystery.

The evolution of drug resistance is an important process, and the resistance to fluconazole, the most widely used antifungal, is often associated with acquired aneuploidy (Selmecki *et al.*, 2009). Selmecki *et al.* (2009) conducted a longitudinal

study of the prevalence and dynamics of chromosomal rearrangements, including aneuploidy, in the presence and absence of fluconazole during *in vitro* evolution experiments using *Candida albicans*, the most prevalent human fungal pathogen. They performed contour-clamped homogenous electric field karyotype analysis followed by Southern hybridization to detect gross chromosomal rearrangements, along with comparative genome hybridization analysis to detect alterations in chromosome copy number (Selmecki *et al.*, 2009). While no aneuploidy was detected in any of the no-drug control populations, in all fluconazole-treated populations analyzed an isochromosome 5L [i(5L)] appeared soon after exposure to fluconazole. This isochromosome is composed of two identical chromosome arms (Chr5L) flanking the centromere. This isochromosome was associated with increased fitness in the presence of fluconazole and, over time, became fixed in independent populations. Fitness was measured by reproductive outputs of clones that contained i(5L) and those that did not contain i(5L). Furthermore, the growth rate and maximum cell density achieved by strains containing i(5L) was much higher than that of sibling strains lacking i(5L) (Selmecki *et al.*, 2009).

Other aneuploidies, particularly trisomies of the smaller chromosomes (Chr3-7), appeared throughout the evolution experiment, and the accumulation of multiple aneuploid chromosomes per cell coincided with the highest resistance to fluconazole (Selmecki *et al.*, 2009). This can be partially explained by the gene *NCP1*, which encodes NADPH-cytochrome P450 reductase and is a co-factor of Erg11p (the target of fluconazole) in sterol 14 alpha-demethylation in ergosterol biosynthesis. *NCP1* is located on Chr4, which was one of the aneuploid chromosomes observed, and therefore

extra copies of Chr4 may provide increased levels of resistance to fluconazole because of the increased levels of *NCP1* (Selmecki *et al.*, 2009).

Conclusions

Mendelian inheritance is based on genetic information known to be contained within the genomes of parental strains, which is then segregated and independently assorted into offspring during sexual reproduction. It is also relevant only to circumstances of perfect meiosis. But how would Mendel account for genetic information not known to be contained within either parent, yet miraculously is found in progeny isolates? How would he account for imperfect chromatid segregation/separation at anaphase? And how could he make a prediction if supernumerary chromosomes were prominently dispersed throughout the experimental population? The answers are simple; he couldn't! But these cases are a minority. Mendel's laws are part of the foundation that make up the field of Genetics, and like any other law or tenet, have faced and will continue to face scrutiny and opposition. Just because observations are made and proven that contradict Mendelian segregation does not mean that Mendel's laws are not true, it just means that the processes of Meiosis are imperfect.

The detection of aneuploidy in fungal pathogens isolated directly from patients and from environmental samples suggests that variations in chromosome organization and copy number are a common mechanism used by pathogenic fungi to rapidly generate diversity in response to stressful growth conditions, including antifungal drug exposure. This has led to the concept of genomic plasticity in certain fungal species

such as *Candida albicans* (Selmecki *et al.*, 2009). The remainder of this review will be devoted to exploring this concept of genomic plasticity in *A. flavus*.

Future research

GENOMIC PLASTICITY IN *ASPERGILLUS FLAVUS*

Introduction

Aspergillus flavus has a wide host range and is one of the few, if not the only known fungal pathogen of both plants and animals. Not only can this fungus infect a wide variety of host organisms, it also possesses the capability of producing carcinogenic aflatoxins (AFs) that affect humans and animals. The destructive properties of this fungus are not in the effects of this fungus on the development processes of its host crop, rather, entire fields are plowed over once the maximum tolerable specification of AF concentration is reached. With the discovery of the sexual cycle in *A. flavus* in 2009 (Ehrlich, Cotty, 2004), current studies now aim to elucidate the purpose of sex, or the inability to do so, in filamentous ascomycete fungi and the implications thereof, such as mycotoxin heritability, nuclear migration, heterokaryon incompatibility, etc. Many aspects of this organism have been genetically characterized in great detail; for example, the AF biosynthetic pathway (Yu *et al.*, 2005), yet the most fundamental of processes that pertain to this fungus (e.g., virulence factors and other pathogenicity determinants) remain unclear. Microbial plant pathogens secrete proteins (e.g., effectors) to acquire nutrients and modulate plant defenses in order to successfully invade and colonize host tissue. To counter this attack, plants have evolved resistance (R) proteins for the recognition of specific pathogen effectors, resulting in a plant defense response known as effector-triggered immunity. Thus, effector proteins that

have evolved to conduct virulence functions can themselves become recognition factors and, consequently, alteration or loss of effector genes enables pathogens to circumvent detection (Catanzariti, Jones, 2010). This creates an ongoing evolutionary battle of attack and counter-attack, best known as the red queen hypothesis (Dasgupta *et al.*, 2005). The recognition of effectors by R proteins occurs both directly through physical contact and indirectly via specific changes in the host cell due to the action of the effector (Catanzariti, Jones, 2010). Instead of investing millions, if not billions of dollars in research and development of biocontrol products that aim to displace the AF producing strains in nature, the answer to an ultimate means of control may lay in better understanding the processes that govern the initial interactions between the host and the pathogen. This proposal aims to make use of data readily available (e.g., genome sequences) to delve into an area of research waiting to be explored.

The genomes of representative strains of different *Aspergillus* species have been sequenced, including: *A. flavus* strain NRRL 3357, *A. oryzae* strain RIB40 (Machida *et al.*, 2005), *A. fumigatus* Af293 (Nierman *et al.*, 2005), and *A. nidulans* strain FGSC A4 (Galagan *et al.*, 2005). In order to scavenge for the gene regions responsible for producing the elusive effector proteins involved in infection and pathogenicity in *A. flavus*, I propose to compare the sequenced genomes of *A. flavus*, *A. oryzae*, *A. fumigatus* and *A. nidulans*.

A. flavus and *A. oryzae* both have the ability to infect plant hosts, whereas *A. fumigatus* and *A. nidulans* do not. Excluding the conserved sequences between *A. flavus*, *A. fumigatus*, and *A. nidulans* and focusing on the conserved sequences between *A. flavus*

and *A. oryzae*, the pool of potential candidates that may be involved in infection or virulence in *A. flavus* towards plant hosts will be narrowed down. To that respect, the vice versa methodology may be used to scavenge for effectors involved in infection and/or virulence in *A. flavus* towards animal and human hosts. Within the pool of potential candidates, further weeding out may then be done by searching against the list of candidates with known protein sequence motifs of fungal effector proteins (Catanzariti, Jones, 2010; Oliva *et al.*, 2010).

Once several strong candidates are identified, deletion mutation constructs can then be made in order to study the genes via reverse genetics. If an altered phenotype results with the deletion of a gene, a wild-type copy of the gene can then be inserted back into the mutant strain to check if the original phenotype can be restored. The overall goal of this research is to identify and characterize virulence and effector proteins of *A. flavus* that are involved in pathogenesis of corn. This would require at least three different steps. First, the phenotypes of each of the selected strains would be determined on living subjects of both mice and corn. Second, a genome comparison of the four strains being used will be done in order to narrow the field of potential genes producing effector proteins to those that are common between *A. flavus* and *A. oryzae*, but dissimilar to those of *A. fumigatus* and *A. nidulans*. Finally, once several putative genes are selected for, deletion analysis will be performed to determine the functionality of each of the genes selected.

Phenotype analysis

In a classic study done by Ford and Friedman in 1967, mice were intravenously infected with spores of several members of the genus *Aspergillus* to determine virulence. They found that all of the species tested, which included *A. flavus*, *A. oryzae*, and *A. fumigatus*, except for a few, which included none of the ones aforementioned, killed mice when 10^6 spores were inoculated intravenously (Ford, Friedman, 1967). Other studies (Bignell *et al.*, 2005; Chang *et al.*, 1998) have tested the virulence of *A. nidulans* on mice and have shown that *A. nidulans* can cause mortality shortly after infection. *A. flavus* is well known as being a pathogen of corn (Leger *et al.*, 2000). Host specialization was tested for *A. flavus*, *A. nidulans*, and *A. fumigatus* and found that *A. flavus* had a lack of host specialization, being able to infect bean leaves, corn kernels, and insects; however, *A. nidulans* and *A. fumigatus* failed to invade living insect or plant tissues (Leger *et al.*, 2000). The spore solutions of all four *Aspergillus* species being tested would be intravenously injected into 8-11 wk old mice subjects and corn kernels. After several days of incubation, results will indicate whether either of the *Aspergillus* spp. were pathogenic to either mouse or corn. In this proposal, the sequenced strain of *A. flavus* (NRRL 3357) was isolated from a plant and therefore the possibility remains, through evolutionary processes, this isolate may have evolved to no longer contain certain genes coding effector proteins specific for pathogenicity toward animals; these preliminary experiments are being done to make certain that the representative strain of *A. flavus* is pathogenic on both mice and corn.

Genome comparisons

The genome sequences of *A. nidulans* (Galagan *et al.*, 2005), *A. fumigatus* (Nierman *et al.*, 2005), and *A. oryzae* (Machida *et al.*, 2005) have been published, and that of *A. flavus* is in the draft stage. A study that compared the genomes of several *Phytophthora* species (Haas *et al.*, 2009) revealed insights into the localization and predicted activities of genes that are induced during infection; I propose to do similar comparisons for the afore mentioned *Aspergillus* genomes. This step would utilize information that is readily available and very little, if any, additional sequencing would be required. Depending on the outcome of the previous objective (i.e., phenotypic analysis), the conserved sequence partitions between the genomes of those species that are found to be pathogenic only towards mice will be compiled and subtracted from the *A. flavus* genome database. Rationale for this step would be to remove sequence motifs of protein encoding genes that may be involved in pathogenicity towards non-corn subjects. Furthermore, the conserved sequence partitions between the genomes of those species that are found to be pathogenic only towards corn or towards corn *and* mice, will be maintained and all other extraneous sequence information removed.

Not very many effector proteins in filamentous plant pathogens have been described, but motifs are available of those that have been characterized (Oliva *et al.*, 2010). A search against the database of remaining protein-coding sequences with all of the known sequence motifs of known effector proteins will further narrow down any potential genes within *A. flavus* that may have a role in pathogenicity.

Deletion constructs and mutant phenotype analysis

Once several candidate virulence factor or effector encoding genes have been identified, characterization of these genes will be done by creating deletion mutants of *A. flavus* strain NRRL 3357, and the phenotypes of these mutants will be assayed on corn kernels to determine pathogenicity or lack thereof. Deletion constructs will be made and transformation procedures will be done following the methods of Chang et al. (Chang *et al.*, 1998). Corn inoculation will follow the procedures as previously described in my first objective. At the point at which a mutant is found to no longer be pathogenic on corn, a wild-type copy of the gene can then be reinserted into the mutant in hopes that the wild-type phenotype will be rescued.

Expected results and further research

A. oryzae, *A. fumigatus*, and *A. nidulans* share about 66-70% sequence similarity (Galagan *et al.*, 2005), which isn't an extraordinarily high degree, but if *A. flavus* shares a high sequence similarity to that of *A. oryzae*, which it is expected to have, eliminating ~70% of the sequence information (i.e., sequence information pertaining to *A. fumigatus* and *A. nidulans*, which are expected to only be pathogenic on mice and not corn) is a large component of the *A. flavus* genome. Furthermore, removing non-conserved sequence information between *A. flavus* and *A. oryzae* (i.e., sequence information proposed to not have any purpose in pathogenicity because both are expected to be pathogenic on corn) will narrow down the list of candidate effector/virulence protein encoded gene regions even more. In addition, screening for

motifs of effector and virulence protein encoded gene regions will greatly weed-out any superfluous information.

This research will garner a greater understanding of *A. flavus*' ability to infect a wide host range, which encompasses several kingdoms of the Tree of Life. In the preliminary phenotype assays on these *Aspergillus* spp. isolates, a concern is that the *A. flavus* strain, which was isolated from a plant would no longer be pathogenic on animals; however, if the *A. flavus* isolate is found to be pathogenic on both mice and corn, hypothetically, virulence factors towards both mice and corn are being maintained within the genome of this *A. flavus* isolate, and continuation of this proposal will reveal *Aspergillus* pathogenicity factors. In the case that the *A. flavus* isolate is not pathogenic on mice, this would suggest that the plasticity of the *A. flavus* genome has evolved to maintain pathogenicity on plants, and perhaps genome rearrangements or conditional loss of pieces of genomic information has been lost. An alternative approach to finding unique sequence information, which could include effector genes, specific for pathogenicity on animals or plants is to compare the genomes of clinical isolates of *A. flavus* to those that were isolated from a variety of plants (e.g., corn and peanut). This comparison could reveal potential genomic reconstruction, copy number variation, gene conversion, or even aneuploidy that are resultant of evolutionary processes that make a fungal lineage specific for animals and another lineage specific for animals.

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