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The Population Dynamics of Aflatoxigenic Aspergilli

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1. Introduction

A. flavus is a saprophyte that possesses the characteristics of an opportunistic pathogen with a wide host range (plants and animals) (Wilson, Mubatanhema et al. 2002). This fungus has substantial impact on agriculture because it produces aflatoxins (AFs), the most toxic and carcinogenic of all mycotoxins (Cary, Bhatnagar et al. 2000). Contamination of susceptible crops with AFs leads to serious health risks in developing countries and significant economic losses in the U.S. and other developed countries (Yu, Payne et al. 2008). Major commodities affected by AFs include corn, cotton, peanuts, tree nuts, rice, peppers, spices, and figs. AF contamination of crops usually results from opportunistic invasion due to some type of injury to the plant. In most infestations, the fungus causes little overall damage to the plant or its fruiting structures. Because *A. flavus* does not produce true plant pathogenicity factors, infection depends on both the plant's internal defenses and the ability of *A. flavus* to invade plant tissues. *A. flavus* is one of several known species that produces aflatoxins, including, *A. parasiticus, A. nomius, A. bombycis, A. pseudotamarii, A. ochraceoroseus,* and others. *A. flavus* and *A. parasiticus*, in particular, are disseminated widely in agricultural regions.

2. *A. flavus* diversity

2.1 Sclerotial size

A. flavus as a species contains two morphotypes that differ in sclerotial size and in their ability to produce AFs. Large (L) and small (S) sclerotial strains are often found in soils from both agricultural fields (Cotty, Bayman et al. 1994; Horn 2007) and non-agricultural areas (Ehrlich, Kobbeman et al. 2007) throughout the world. Recently, it was suggested that the S- strain, which is capable of producing much higher concentrations of aflatoxins than the L- strain, may be a more important source of aflatoxin contamination in corn and cotton in some regions (Zhang and Cotty 2007). Beyond sclerotial size, another difference between the L- and S- strain is colony morphology, since S- strain isolates produce many more sclerotia and, in the dark,

fewer conidia (Cotty, Bayman et al. 1994). Furthermore, S- strain isolates have a higher virulence toward cotton as measured by production of pectinase (Mellon and Cotty 2004). The specific ability of *A. flavus* morphotypes to adapt to agricultural environments is poorly understood.

2.2 Vegetative compatibility

A. flavus populations are composed of sub-clades consisting of different vegetative compatibility groups (VCGs). Fungal isolates from different VCGs are presumed to have limited genetic exchange and recombination in the parasexual cycle since vegetative compatibility is determined by a series of heterokaryon incompatibility loci whose alleles must all be identical for stable hyphal fusions to occur (Leslie 1993). Populations of *A. flavus* and other section *Flavi* species are complex and consist of many VCGs (Bayman and Cotty 1991; Horn, Greene et al. 1995). The number of VCGs in a field may vary based on soil type, climate, and type of crop, and may even vary from year to year. No particular VCG has been consistently associated with a specific niche, and it is not clear if the VCG trait is important for adaptation although characters associated with VCG may be important. VCG analyses have shown that genetically distinct *A. flavus* strains frequently interact during dispersal, growth, and crop infection (Bayman and Cotty 1991; Cotty, Bayman et al. 1994). Isolates from different VCGs often vary in their ability to produce AF (Bayman and Cotty 1991; Horn, Greene et al. 1995). How strong a barrier vegetative compatibility is to recombination within a fungal species, due to parasexuality, is unknown. A comparison of three genes in *A. flavus* isolates of the same VCG performed by Ehrlich et al. (Ehrlich, Montalbano et al. 2007), and array comparative genome hybridization assays by Federova et al. (Federova, Harris et al. 2009) showed that isolates from the same VCG have identical genomes. These results suggest that isolates in the same VCG represent a clonal assembly. A study by Grushiba and Cotty (2009) estimated divergence time between examined VCGs to range from 50,000 to 200,000 years.

2.3 *A. flavus* non-aflatoxigenicity

Non-aflatoxigenic *A. flavus* strains are represented by both domesticated and wild-type forms. Domesticated *A. oryzae* is genetically indistinct from *A. flavus. A. oryzae* is used as a starter culture for the preparation of fermented foods and alcoholic beverages, and is an important source of many enzymes, such as glucoamylase, alpha-amylases and proteases used for starch processing, baking, and brewing worldwide (Machida, Yamada et al. 2008). Wild-type non-aflatoxigenic *A. flavus* is a common inhabitant of soil and crops from agricultural areas worldwide and co-exists with its toxigenic brethren. In agricultural fields, up to 40% of the isolates lack the ability to produce aflatoxins due to deletions or mutations within the aflatoxin cluster (Cotty and Bhatnagar 1994; Chang, Horn et al. 2005). Non-aflatoxigenic (AF-) *A. flavus* strains are currently being introduced into agricultural fields as biological control agents to lower crop aflatoxin contamination. Within the non-aflatoxigenic group are isolates incapable of producing the mycotoxin cyclopiazonic acid, while other isolates produce this mycotoxin. Although AF- domesticated forms of *A. parasiticus* are known (e.g. *A. sojae*), AF- isolates of *A. parasiticus* are rarely found in natural environments.

3. *A. flavus* and *A. parasiticus* exhibit evidence of recombination

P.A. Micheli characterized the genus *Aspergillus* as an asexually-reproducing group of fungi in 1729. Nearly a century later the first teleomorph was discovered and named *Eurotium*

herbariorum by J.H.F. Link, which Anton DeBary later identified as the sexual state of *A. glaucus* (Raper and Fennell 1965). Though other teleomorphs have been identified over the years, the genus *Aspergillus* is still considered to be predominantly asexual due to lack of morphological evidence of sex in nature.

Populations of aflatoxigenic fungi were originally considered to result from the clonal amplification of individuals with the same chemotype profile, in contrast to the possibility of creating novel chemotype profiles, or restoring aflatoxin production in individuals that are AF-, through genetic exchange and recombination (Geiser, Pitt et al. 1998; Pitt and Hocking 2006). The belief that the aflatoxigenic Aspergilli reproduce asexually was the foundation for trusting in the stability of non-aflatoxigenic biocontrol strains in competitively excluding indigenous aflatoxigenic Aspergilli in fields (Geiser, Pitt et al. 1998; Moore, Singh et al. 2009). Currently there are two U.S. Environmental Protection Agency-approved biocontrol strains, both species of *A. flavus*, who are unable to synthesize aflatoxins due to either mutations in aflatoxin-synthesis pathway genes, or the loss of those genes altogether. A cryptic sexual state was reported in aflatoxigenic (AF+) *A. flavus* suggesting a history of recombination (Geiser, Pitt et al. 1998); therefore, the possibility of genetic exchange and recombination within populations of AF+ fungi may necessitate a re-evaluation of biocontrol selection strategies.

As molecular research to understand, and subsequently prevent, contamination by aflatoxigenic fungi has evolved, the *Aspergillus* research community has begun to explore population structure of AF+ Aspergilli. The tools to observe and interpret population dynamics are also evolving, beyond comparisons of mutational differences, allowing for stronger inferences regarding the mechanisms that maintain functional and genetic diversity in AF+ fungi. This chapter will review population genetics methods employed to obtain evidence of recombination within populations of two of the more common agents of aflatoxin contamination in agricultural commodities, *A. flavus* and *A. parasiticus*; the subsequent (current) research developments that support inferences made regarding recombination in these fungi; and where the investigations are leading researchers who continue to seek protection for the global food supply.

3.1 How is recombination detected in a population?

Recombination can be defined as the exchange of genetic material between individuals at the chromosomal level, oftentimes resulting in novel genomic compositions in offspring, since chromosomes from both "parents" are being broken apart and shuffled around during meiosis (Milgroom 1996). Detecting recombination depends on the amount of allele shuffling that is occurring in a population. If there is high genetic diversity and frequent shuffling, then detection is easier; conversely, low genetic diversity accompanied by little to no shuffling of alleles, might indicate no evidence of recombination (Nordborg and Tavaré 2002). Figure 1 illustrates how recombination may be detected in two simplified populations. Both populations are composed of the same sample size, and each population shares the same sequence breakpoints (X), with uniform recombination rates, between three contiguous loci. The nucleotide bases are color-coded blue or red with random mutations colored black. Population 1 has an approximately equal distribution of blue and red alleles that are shuffled around sequence breakpoints. Population genetics tools would easily detect recombination within this population. Population 2 appears clonal because of a predominance of the red allele, perhaps because it confers some selective advantage; conversely, the blue allele is found in one individual which may be a

recent migrant, the survivor of a recent bottleneck, or a more fit individual under certain environmental conditions and therefore maintained, at a low frequency, in the population via balancing selection. The power of population genetic tools to detect evidence of recombination for population 2 is limited since there is not as much shuffling (or mixing) of both alleles. Increasing the sample size of population 2 may or may not alter the results. One benefit to studying a non-recombining population is the potential to detect phenotypic targets of balancing selection in the genomes of its individuals (Carbone, Jakobek et al. 2007; Moore, Singh et al. 2009). Though selection may be acting on all three loci, our ability to observe evidence of the phenotypic targets of selection depends on finding a region that is not subject to frequent shuffling which would obscure genotype-phenotype associations.

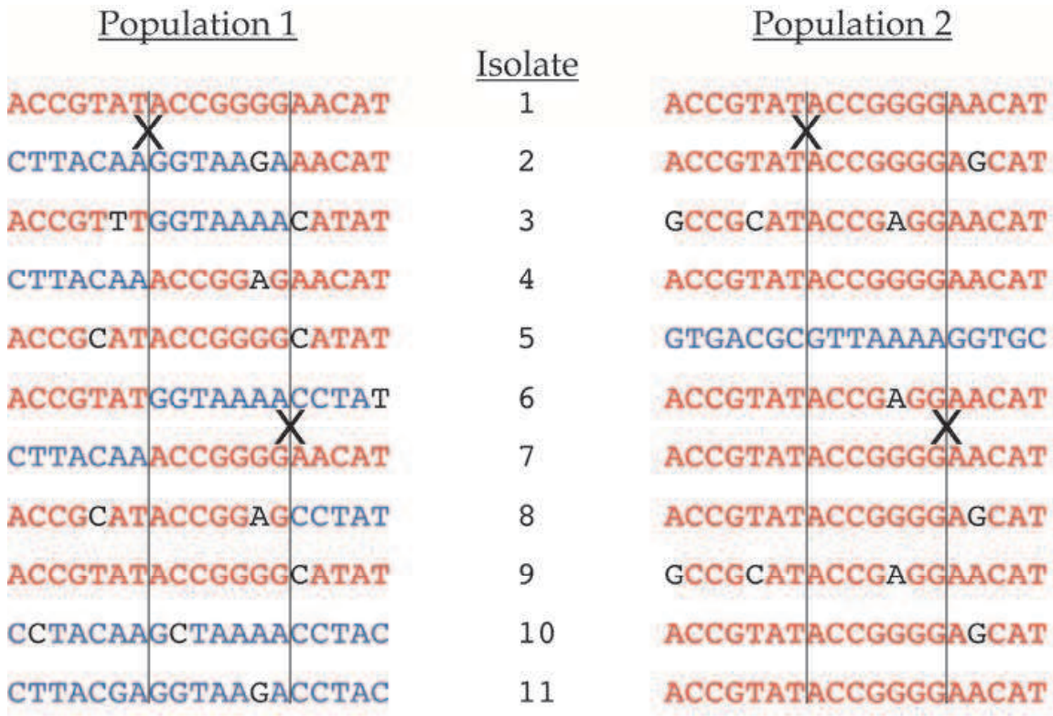

Fig. 1. Patterns of recombination among three concatenated linked loci in two isolated populations; loci boundaries are delineated using vertical lines.

3.2 Population genetics tools

A plethora of genetics tools are available for population analysis. These can be grouped into three broad categories: 1) non-parametric and summary statistic methods, 2) genealogical estimators of population parameters, and 3) coalescent and Bayesian approaches for estimating the direction and magnitude of evolutionary forces.

Non-parametric and summary statistic methods are frequency-based. These non-genealogical methods follow a simple model known as Hardy-Weinberg equilibrium which assumes only Mendelian inheritance and no effect on populations from evolutionary forces; so as long as the assumptions are not violated, allele frequencies will persist in a constant state with each generation (Hartl and Clark 1989; Hey and Machado 2003). Methods such as

Classical Wright's FST statistics, neutrality tests (Tajima 1989; Fu 1996), population subdivision (Hudson 2000), population mutation rate estimators (e.g. Watterson's θ), and compatibility methods (Quesne 1982) are frequently examined under a simple model of evolution.

Genealogical estimators of population parameters involve simple populations exhibiting the Wright-Fisher model of random genetic drift (Hey and Machado 2003). This means that every generation has a constant population size, and genetic drift is the only evolutionary force altering allele frequencies. Genealogical approaches can enhance the power of neutrality tests (Fu and Li 1993), haplotype phenotype association testing (Templeton, Routman et al. 1995), and demographic inference.

Coalescent and Bayesian approaches are considered parametric (involve parameters) and follow a complex genealogical model. As previously mentioned, the methods implemented here are for estimating the direction and magnitude of evolutionary forces. Coalescent approaches estimate: θ, recombination, and migration rates (Beerli and Felsenstein 1999). Bayesian inference uses probability distributions to quantify uncertainty (Nielsen and Wakeley 2001; Grünwald and Goss 2011). This includes equilibrium and non-equilibrium models, which relate to continuous versus sporadic processes, respectively.

Several population software tools are available that implement the various methods described above; for a comprehensive review see Grunwald and Goss (2011). Some useful analysis packages are Arlequin (Excoffier, Laval et al. 2005), GENEPOP (Raymond and Rousset 1995) and POPGENE (Yeh and Boyle 1997). Price and Carbone assembled a collection of population analysis programs and named it SNAP Workbench (2005). Imported sequence alignments are collapsed into haplotype groupings as "individuals" using SNAP Map (Aylor, Price et al. 2006) followed by various analyses to detect recombination. Within SNAP, analyses such as linkage disequilibrium (LD), the ancestral recombination graph (ARG), and allele coalescence have been used to detect recombination in populations of *A. flavus* and *A. parasiticus*.

3.2.1 Linkage disequilibrium (LD)

LD is a means to infer distinct evolutionary histories in populations (Nordborg and Tavaré 2002). The power of detecting LD depends on distinct SNP-patterns found in either a single allele or across a group of alleles. An LD plot (Figure 2), is generated based on pairwise measures of LD. Three commonly used LD measures with slightly different properties are D', r^2, and d^2. D' is a normalized value (between 0 and 1) regardless of allele frequencies, r^2 is the squared association in allelic state between two loci existing in haplotypes, and d^2 measures association between alleles for each locus (Nordborg and Tavaré 2002). For example, a typical LD plot is divided into two triangular portions showing all pairwise comparisons in the data. Significant LD is assessed using a simple Fisher's Exact test for a 2 x 2 contingency table or a permutation method if more than two alleles are present. LD can be examined for both single-locus and multi-locus data.

In Figure 2, population 1 (from Fig. 1) harbors three distinct LD blocks, which may translate to distinct evolutionary histories in the data. LD is significant with at least two individuals sharing red and blue alleles; correlation is strong with many polymorphisms segregating for the red and blue alleles. The LD pattern for population 2 shows extensive LD with essentially one large haplotype block. Table 1 lists identities for haplotypes in Figs. 2 and 3, in which we observe higher haplotype diversity in population 1 than in population 2.

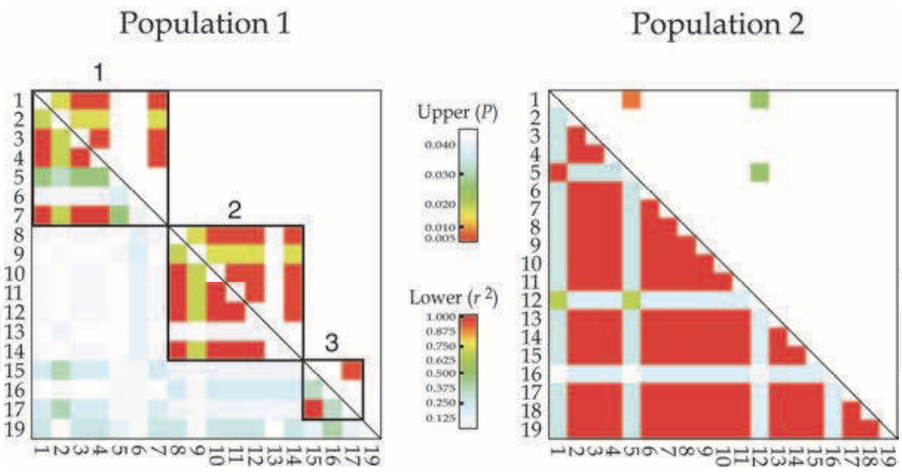

Fig. 2. LD plots based on combined loci for populations in Fig. 1.

Population 1	
Haplotype	Isolate(s)
H1	1
H2	2
H3	10
H4	4
H5	7
H6	11
H7	5
H8	8
H9	3
H10	6
H11	9

Population 2	
Haplotype	Isolate(s)
H1	1, 4, 7, 11
H2	3, 9
H3	5
H4	6
H5	2, 8, 10

Table 1. Haplotype composition for hypothetical analyses shown in Figs. 2 and 3, based on collapsed sequences from Fig. 1.

3.2.2 Ancestral Recombination Graph (ARG)

The analysis software RecMin is often used to determine the minimum number of recombination events in a population (Myers and Griffiths 2003), whereas the ARG illustrates the relationships of homologous sequences undergoing recombination by coupling allele coalescence with recombination events (Lyngsø, Song et al. 2005). Both analyses are based on the four-gamete test that looks for patterns of incompatibility among pairs of sites exhibiting all four gametic types. Lyngsø et al. (2005) consider the ARG to be an improvement on RecMin, especially for large population samples. There are two different parsimony-based algorithms for inference of an ARG: Beagle and Kwarg. Each will attempt to infer the ancestral state from a group of nucleotide sequences whether or not a root is known. The four-gamete test is performed on sequence alignments as sections, reconstructing a history with each pass. Beagle searches for the most parsimonious reconstruction using branch and bound, so it may be limited by complex datasets with a great deal of recombination; therefore, Lyngsø later created a heuristic implementation that

is less exact to handle more complex population datasets and named it Kwarg (http://www.stats.ox.ac.uk/~lyngsoe/section26/). In an ARG, mutations are shown as numbers along its branches (see Fig. 3), assuming a standard infinite sites model. An ARG shows population haplotypes/individuals encircled in red (present time), and the population's history is traced back to the most recent common ancestor at the top (in the past). Recombination events are encircled in blue and show the position of the variable site in the DNA sequence alignment where the breakpoint occurs. Preceding each recombination event are branches labeled with "P" (prefix) or "S" (suffix) that correspond to the 5′ and 3′-most segments, respectively, that are exchanged by a crossover between putative parental lineages. Graph nodes that correspond to coalescence/divergence events are shown as green dots in an ARG; a yellow dot denotes the inferred ancestral (root) sequence.

Using Beagle to infer ARGs, we observe evidence of recombination in the histories of populations 1 and 2 (Fig. 3). Population 1 has experienced recombination throughout its history, and haplotypes H2 and H9 were formed by recent recombination events between two parental haplotypes (H1+H6 and H10+H7, respectively). Population 2 shows only a single recombination event in its history whereby H3 is the putative offspring of H2 and H5. Clearly recombination has given rise to a greater number of haplotypes at these three loci in population 1 since there are eleven haplotypes compared to the five haplotypes for population 2.

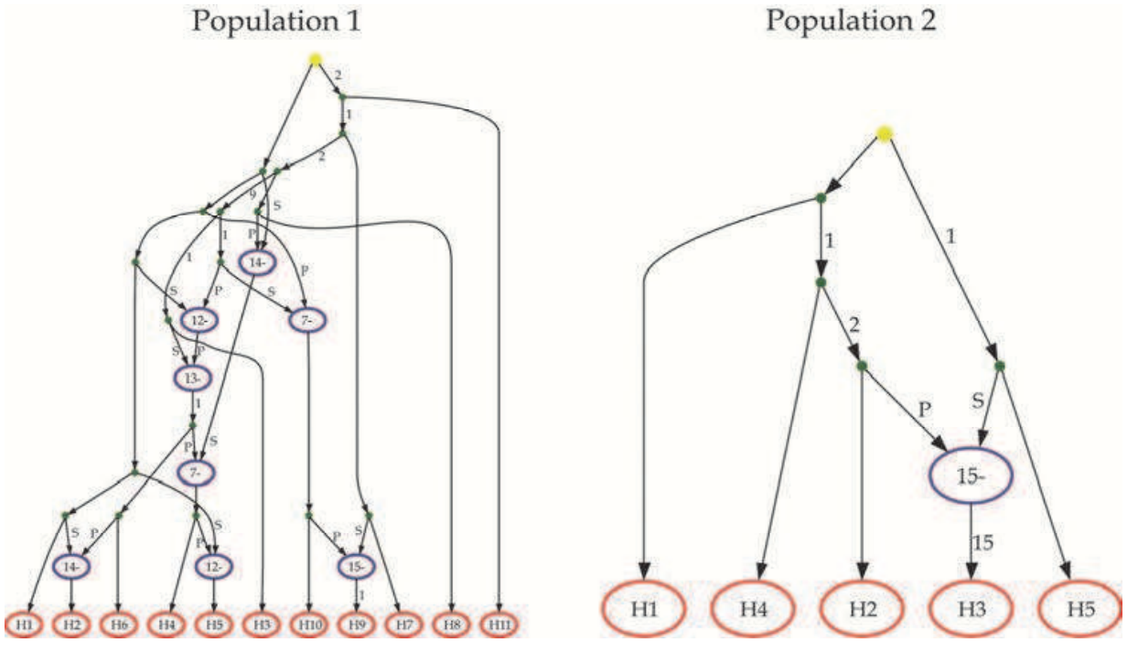

Fig. 3. ARGs based on combined loci for populations in Fig. 1.

3.2.3 Allele coalescence

Allele coalescence is rooted in the coalescent theory, which states that all alleles in a population are inherited from a single ancestor and are shared throughout the population. Coalescence is the reverse of divergence, so two lineages are traced back to their common ancestor. Coalescence may be inferred for a single locus or for multiple linked loci using GENETREE version 9.0 (http://www.stats.ox.ac.uk/~griff/software.html). Gene

genealogies inferred using the coalescent reconstruct mutational relationships either within a "panmictic" population undergoing random mating, or among "subdivided" populations experiencing limited gene flow. Coalescent simulations can be performed assuming constant population sizes or exponential growth (Griffiths and Tavaré 1994). Similar in structure to an ARG, the coalescent will have the haplotypes at the tips of branches (present time) that coalesce to the most recent common ancestor (MRCA) in the past. Mutations are shown as dots along the branches. An interesting feature of the coalescent model is that coalescent time (shown as a scale above the genealogy in Fig. 4) can be converted to real time, giving approximate divergence times, in millions of years. Figure 4 shows examples of gene genealogies inferred for the three combined loci. The numbers in parentheses adjacent each haplotype refer to the isolate numbers from Figure 1.

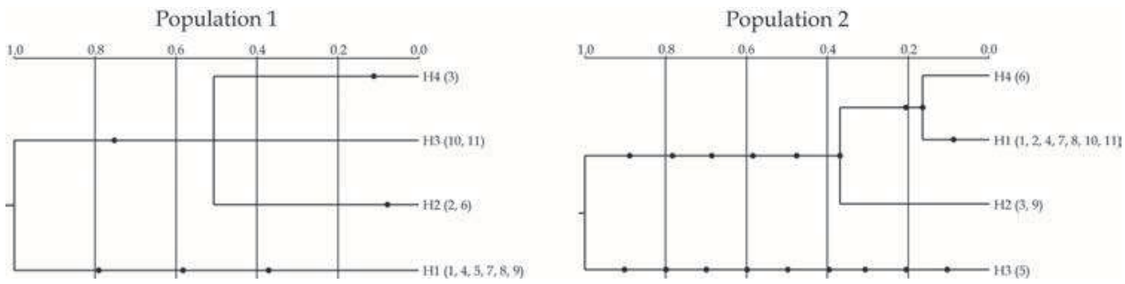

Fig. 4. Coalescent-based gene genealogies based on combined loci for populations in Fig. 1.

An important consideration when inferring gene genealogies, using a coalescent model that assumes no recombination, is that there are no incompatible sites in data. Frequent recombination in population 1 creates site incompatibilities since there may be different ancestral histories for each locus; as a result, only the largest non-recombining partition was examined. This affects inference of haplotype diversity and subsequently we see a reduction in haplotype number from eleven to four. Population 2 has only a single recombination event in its history, meaning fewer incompatible sites are present, so the haplotype number of four is much closer to initial population diversity (five haplotypes).

3.3 A comparison of recombination in *A. flavus* and *A. parasiticus*

3.3.1 Linkage disequilibrium

Carbone, Jakobek et al. (2007) used LD to investigate recombination in a single *A. parasiticus* population from Georgia. Starting with a sample of 76 single-spore isolates, an experimental sample of 24 isolates, each representing a separate VCG, was created (Carbone, Jakobek et al. 2007). Moore and co-workers investigated an *A. flavus* population from the same Georgia field from which they began with 92 single-spore isolates that were consolidated to a subset of 44 based on VCG testing (Moore, Singh et al. 2009). Both studies sequenced most of the intergenic regions across the aflatoxin gene cluster and imported those sequences into the SNAP Workbench (Price and Carbone 2005) for various statistical analyses. For *A. parasiticus*, LD analysis revealed evidence of recombination throughout the cluster by formation of five blocks of recombination (Fig. 1 in Carbone et al. 2007). The *A. flavus* LD, shown in Figure S1 of Moore et al. 2009, revealed six blocks of recombination, spanning contiguous late-pathway genes of the cluster (*aflE-aflO*). Recombination was not detected in the early pathway genes (*aflT-aflE*); however, Moore and co-workers did observe increased levels of recombination in a "hotspot" around *aflP*. Given that some of the blocks span

multiple loci, it was inferred that shuffling a group of genes is as likely as shuffling only one gene. Also, since some of the sequences included exons, it is possible to infer that block junctions are in either the intergenic region or the exon. In comparing rates of recombination based solely on LD patterns, it would appear that *A. flavus* is experiencing similar rates of recombination as *A. parasiticus*. The *A. flavus* LD plot exhibited a more significant block structure across contiguous loci than the *A. parasiticus* LD which showed a less organized block structure intermittently throughout the cluster. Both *A. flavus* and *A. parasiticus* LD analyses showed evidence of balancing selection in a non-recombining region of the cluster (*aflW* and *hypE*, respectively). Both studies provided estimates of the minimum number of recombination events (RecMin) for their respective populations. *A. flavus* appeared to have more than four times as many recombination events as *A. parasiticus* in the same field. The higher rate of recombination was particularly evident in the ARG.

3.3.2 Ancestral recombination graphs

The Beagle algorithm was used for the *A. parasiticus* ARG analysis, but attempts to use Beagle for the *A. flavus* sample proved difficult; therefore, the Kwarg algorithm was used because it can handle more complex datasets. ARG analyses revealed that both *A. parasiticus* (Fig. 2B from Carbone et al. 2007) and *A. flavus* (Fig. 2 from Moore et al. 2009) populations from the same field show a history of recombination, but the *A. flavus* population exhibits a more extensive history of recombination. This is not surprising when one compares both the number of VCGs found for each population and the number of distinct multi-locus cluster haplotypes. *A. parasiticus* had fewer VCGs and distinct haplotypes (24 and 10, respectively) than *A. flavus* (44 and 27, respectively), even though the overall numbers of isolates sampled were comparable. It is well known that increased recombination will contribute to greater population diversity (Milgroom 1996).

3.3.3 Allele coalescence

Carbone, Jakobek et al. (2007) inferred a gene genealogy for a non-recombining region in the aflatoxin cluster of *A. parasiticus*, known as *hypE*. They found evidence of trans-speciation (balancing selection), which was supported by the separation of two groups of distinct chemotype classes (see Figure 3 in Carbone et al. 2007). Those distinct chemotype classes were reportedly related to G- and B- aflatoxin production versus *O*-methylsterigmatocystin (OMST) production. For the *A. flavus* study, Moore, Singh et al. (2009) performed coalescent analysis for a non-recombining cluster region known as *aflW* (*moxY*). They observed evidence of balancing selection for this region, and once again the genealogy (Fig. 5 in Moore et al. 2009) supported separation of the population into two groups based on distinct chemotype classes. In *A. flavus*, balancing selection appears to be between AF+ and AF- individuals because most full-cluster, and all partial-cluster, AF- isolates grouped as a separate clade/lineage from that of the AF+ isolates.

Both non-recombining loci offer glimpses into the ancestral states for each species, and perhaps the basis for balancing selection to maintain the ancestral states is cohesiveness of functional units. A plausible explanation for these results is that the gene *hypE*, and its cluster neighbor *aflN* (*verA*), encode proteins that catalytically function as a unit. Balancing selection would favor keeping these genes together in an evolutionary shuffling process. This suggests that, by a process not yet fully understood, the protein encoded by *hypE* encodes a helper protein that is required for the proper function of the cytochrome P450 monooxygenase, *aflN*, the first enzyme needed for conversion of

versicolorin A to sterigmatocystin. Similarly, both *aflV* (*cypX*) and *aflW* (*moxY*), the oxidative enzymes required for conversion of averufin to versicolorin B, also function as a unit so balancing selection maintains these as non-recombining loci (Kenneth C. Ehrlich, unpublished).

3.4 Additional inferences resulting from recombination analyses

Moore, Singh et al. (2009) explored the role of G+C content in *A. flavus* recombination, on chromosome III, from the telomere to the centromeric end of the aflatoxin gene cluster. For Fig. 3 in Moore et al. 2009, a *z'* curve (Zhang and Zhang 2004) is shown that illustrates a steady increase of G+C content, from the telomere to the *aflF* (*norB*) gene, seen as a negative slope. The curve then rises sharply, as G+C content decreases, until the *aflE* (*norA*) gene where it plateaus to *aflQ* (*ordA*). G+C content begins to increase toward the end of the cluster, seen again as a negative slope of the curve. When one considers the lesser effort it takes to break apart an A+T [double] bond, then shuffling of genetic material might be easier to accomplish in genomic regions where the G+C content is lowest (Zhang and Zhang 2004). Perhaps the G+C content in *A. parasiticus* exists at higher levels than in *A. flavus*. Earlier it was mentioned that sequence breakpoints may exist in either the gene or the intergenic region. Moore et al. explored this concept by performing LD analysis on four complete cluster sequences. In Figure S2 from Moore et al. 2009, there was evidence of sequence breakpoints existing in both gene and intergenic region for *A. flavus* (AF13, AF36, and NRRL 3357) and *A. oryzae* (NRRL 5590). Therefore, even a single gene might have multiple histories if it has been experiencing frequent shuffling. Another finding worth noting pertains to the relative ages of partial-cluster to full-cluster AF- *A. flavus* strains. Referring once again to the gene genealogy from Fig. 5 in Moore et al. 2009, it appears the partial-cluster strains are recently derived from full-cluster ancestors, offering evidence of gene loss instead of gain. This suggests that partial-cluster strains are an evolutionary dead-end and that such strains may offer a more stable foundation as biocontrol agents.

3.5 Evolution of the aflatoxin gene cluster

Gene clusters that contain syntenic AF/sterigmatocystin (ST) biosynthesis genes have been found in very diverse fungi, including *Mycosphaerella pini*, the causative agent of needle blight in pine, (Bradshaw and Zhang 2006); *A. terreus*, a species commonly found in agricultural fields (Carbone, Ramirez-Prado et al. 2007); and the human pathogens *A. fumigatus* (Carbone, Ramirez-Prado et al. 2007) and *Coccidioides immitis* (Sharpton, Stajich et al. 2009; Ehrlich and Yu 2010). In *M. pini*, the genes encoding proteins necessary for production of the starter fatty acid hexanoyl CoA, HexA and HexB, and the decorating enzymes, OrdB, AvnA, HypC and Vbs, are in a separate cluster from the genes encoding polyketide synthase (PksA) and the decorating enzymes CypX, AvfA, EpoA (not found in AF or ST gene clusters) and MoxA (Cary and Ehrlich 2006). The latter two enzymes are involved in versicolorin A metabolism in AF/ST-producing *Aspergillus* species and are related to genes involved in melanin production in fungi. The regulatory proteins AflR and AflJ are also in a separate locus on the chromosome. Phylogenetic analysis revealed that the PksA types known to accept hexanoyl CoA as the starter unit have a different evolutionary history from other types of polyketide synthases (Ehrlich and Yu 2010).

Aspergillus species that produce both B and G aflatoxins, such as *A. parasiticus*, diverged prior to *A. flavus* (Cary and Ehrlich 2006). Loss of the ability to produce G-type AFs

involves different mutations of the genes encoding enzymes required for G-toxin formation. There is evidence that loss of G AF production occurred at least three separate times among extant *Aspergillus* lineages. Phylogenetic evidence suggests that species with the *Flavus*-type gene cluster diverged from species with the *Nidulans*-type cluster about 75 Mya (Berbee and Taylor 2001; Heckman, Geiser et al. 2001; Kasuga, White et al. 2002). High identity between *Nidulans*- and *Flavus*-type cluster genes reflects purifying selection during divergence as measured by the ratio of rates of non-synonymous (Ka, nucleotide changes that result in amino acid changes) to synonymous nucleotide changes (Ks, nucleotide changes that result in conservation of amino acids) (Foster 2000). The Ka to Ks ratio was much lower for *ver-1* (ratio = 0.05) than for any of the 25 other *Flavus*-type cluster genes (ratio range = 0.2 to 1.04), suggesting that the most intense purifying selection was at this locus. Significantly higher Ka/Ks values in section *Flavi* compared to non-section *Flavi* species are evidence of adaptation and increased positive selection acting on genes in *Flavus*-type clusters with only partial gene sets. The gene *ver-1* encodes a reductase that is remarkably similar to the reductase required for melanin production. This reductase and *ordB* encode enzymes similar to the enzymes required for appressorium formation, a hardened mycelial structure needed for initial infection of plants and insects (Henson, Butler et al. 1999; Inagaki, Takano et al. 2000). This relationship suggests that AF production and other processes geared to offensive strategies for food acquisition by fungi arose from similar genetic roots. Therefore, while extant species of AF-producing Aspergilli are entirely saprophytic, they must have had, at one time, a more aggressively invasive lifestyle.

In the *Flavus*-type cluster there is considerable evidence that gene duplication is involved in cluster evolution (Carbone, Ramirez-Prado et al. 2007). Possible duplicated genes encode the following proteins (amino acid identity is in parentheses): the cytochrome P450 monooxygenases, VerB and AvnA (39%), NorA and NorB (50%), HypC and HypB (42%), and OrdB and AvfA (30%). Additional evidence for gene duplication is that after knockout of certain cluster genes, the resulting mutants still produce AF in addition to precursor intermediates. This "leaky" phenotype is found with the following knockout mutants: *nor-1, adhA, hypB1* or *hypB2, norA, norB,* and *ordB*. This suggests that gene homologs inside or outside of the cluster encode proteins that are able to substitute for the catalytic function of these *Nidulans/Flavus*-type cluster proteins (Ehrlich 2009).

Why are gene clusters maintained? Most fungal gene clusters represent regulatory islands within a region of chromatin that is at least partially heterochromatic (inactive and not able to be transcribed). It has been shown that heterologous genes inserted into the *Flavus*-type cluster are expressed under the same induction conditions as genes that are components of the cluster, whereas cluster genes inserted at loci removed from the cluster are severely down-regulated (Chiou, Miller et al. 2002; Keller, Bok et al. 2006). Many secondary metabolite clusters are in subtelomeric regions of the chromosome (regions generally rich in heterochromatin) (Galagan, Calvo et al. 2005; Wong and Wolfe 2005). Subtelomeric regions are particularly prone to chromatin modification. They tend to have an abundance of duplicated genes as well as retrotransposons and retrotransposon remnants. Such elements indicate a past history of DNA deposition from other locations in the genome (Robyr, Suka et al. 2002). Subtelomeric regions contain genes that permit growth under stress conditions and on unusual nitrogen and carbon sources, but which normally are silenced (Wong and Wolfe 2005). The small *aflR/aflJ* cluster in C. *immitis/posadasii* is associated with a retrotransposon.

After the ancestral cluster formed, gene losses occurred. The *Flavus*-type cluster is missing several *Nidulans*-type cluster genes (Ehrlich and Yu 2010). These genes, *stcC, stcD,* and *stcT*, lack promoter AflR-binding sites, and therefore, are probably not regulated by AflR during ST biosynthesis. They encode proteins with unknown functions and probably are not necessary for ST biosynthesis. A homolog to *stcC* is found in the dothistromin biosynthetic cluster (Bradshaw, Bhatnagar et al. 2002). Another gene loss appears to have occurred relatively recently, perhaps within the last 1 million years. This involves a deletion of the promoter region of the cytochrome P450 monooxygenase, *cypA*, a gene necessary for formation of AFG_1 (Ehrlich, Chang et al. 2004). Two types of deletion are found in *A. flavus*: a 1.2-kb deletion in *A. oryzae* and *A. flavus* S- strain, and a 0.8-kb deletion in most L- strains of *A. flavus*. It is possible that loss of G-toxin production was an adaptive change that permitted variant *A. flavus* to grow more readily on living plant tissues as opposed to being predominantly a resident in the soil.

The genes for AF/ST/dothistromin synthesis have been retained for greater than 125 million years and it is probable that the polyketides encoded by these clusters enabled the fungi to survive and prosper as free-living organisms in diverse environments (Bradshaw, Bhatnagar et al. 2002; Gomez and Nosanchuk 2003). We assume that the reason for maintaining such gene clusters is that the precursor metabolites are critical for fungal adaptation. Primordial fungi probably had a basal polyketide biosynthesis cluster that consisted of genes encoding only a PKS and a few enzymes capable of stabilizing the nascent polyketide. Such a basal cluster could have allowed fungi to synthesize aromatic, colorful molecules that, like plant pigments, may have aided spore dispersal (Cary and Ehrlich 2006). The colorful AF precursors, naphthoquinones and anthraquinones, are found in many types of fungi, including genera that preceded divergence of the Aspergilli. At this stage of cluster evolution, rather than being toxins, these metabolites may have been attractants (Cary and Ehrlich 2006). As the fungi dispersed into less hospitable niches, a need may have arisen for them to be competitive with associated microflora or they may have benefited by acquiring the ability to become virulent to plants (Cary and Ehrlich 2006). Such competitive conditions may have selected for the duplication and adaptation of genes to allow elaboration of an even greater variety of metabolites. There is increasing evidence that AF/ST biosynthesis and conidiospore development are regulated by a shared signal transduction mechanism (Hicks, Yu et al. 1997; Calvo, Wilson et al. 2002; Wilkinson, Ramaswamy et al. 2004).

4. *A. flavus* and *A. parasiticus* have sexual (teleomorphic) states

4.1 Sexuality in fungi

Many fungi have the ability to live a stable existence in either the haploid or diploid state. In yeast, the question was addressed concerning whether haploidy or diploidy confers an evolutionary advantage (Zeyl, Vanderford et al. 2003; Zeyl 2004). Conventional wisdom suggested that having an extra gene copy would prevent harmful mutations from accumulating in the genome whereas in haploid species, harmful mutations would be detrimental to the organism. However, adaptation depends on the rate that the mutation becomes "fixed" in the population. In haploid organisms, harmful mutations are rapidly lost and beneficial mutations are retained, while in diploid organisms, harmful and beneficial mutations take longer to become fixed in the population. Evolution in large populations is dominated by the accumulation of multiple mutations of moderate effect (Desai, Weissman

et al. 2007). In wild populations of yeast, maximum genetic diversity was found in diploids (Ezov, Boger-Nadjar et al. 2006). Genetic diversity however, is only useful if selective pressure requires adaptation. Within an agricultural field, the demand for diversity may be less because a rich source of nutrients is readily available.

4.2 Identification of *MAT* loci in section *Flavi*

Until 2008, the only species in Section *Flavi* that had a known sexual state was homothallic *A. alliaceus* (teleomorph: *Petromyces alliaceus*). Little was known about the genetic structure of the mating-type locus in *A. alliaceus* or whether a mating-type locus existed for AF+ species like *A. flavus* and *A. parasiticus*. With evidence that both of these agriculturally important Aspergilli have histories of recombination, the Carbone lab began exploring the genomes for a *MAT* locus in all three species. Ramirez-Prado, Moore et al. (2008) published evidence of a heterothallic mating system in *A. flavus* and *A. parasiticus* by the amplification of a single idiomorph locus (chromosome VI) that was either *MAT1-1* (alpha-box) or *MAT1-2* (HMG). The *A. alliaceus* mating-type locus contained both idiomorphs on the same chromosome as shown in Fig. 1A from Ramirez-Prado, Moore et al. 2008. A diagnostic test was created to identify the mating-types of the isolates from the Georgia *A. flavus* and *A. parasiticus* populations previously studied.

4.3 *Aspergillus* mating studies

Once the *MAT* loci had been determined for populations of *A. flavus* and *A. parasiticus*, "parental" isolates were selected and paired for evidence of sexual reproduction (Horn, Moore et al. 2009; Horn, Ramirez-Prado et al. 2009). Parents were selected as belonging to both an opposite mating type and a different VCG. Resulting crosses offered morphological evidence of sex through the production of cleistothecia and ascospores. The teleomorphs within section *Flavi* were assigned to the genus *Petromyces* based on the morphology of the sexual state in which cleistothecia are borne within the matrix of stromata, which resemble the sclerotia produced by many section *Flavi* species. Since 2008, the teleomorphs of three AF+ species in section *Flavi* have been characterized: *P. parasiticus* (Horn, Ramirez-Prado et al. 2009), *P. flavus* (Horn, Moore et al. 2009), and *P. nomius* (Horn, Moore et al. 2011).

4.4 Potential impact of sex on population diversity

The greatest potential impact of sex on populations of AF+ fungi concerns the stability of biocontrol strains (Geiser, Pitt et al. 1998; Moore, Singh et al. 2009). What would we expect if an indigenous AF+ strain has sex, and exchanges genetic material, with a biocontrol strain? Tests are underway to determine if any possible offspring from such crosses differ from parents in the production of AFs. Mating in fungi allows for genetic modification to circumvent unfavorable conditions (Lee, Ni et al. 2010). When fields are inundated with a biocontrol strain, AF- genomes may become "rescued" by genetic material from an AF+ parent. Also, population chemotype diversity may increase when sex occurs in fields. One might expect, in an actively recombining population, a gradient of chemotype profiles. For instance, in *A. parasiticus* populations, there may be less distinction between G- or B-dominant isolates, i.e. more of the sample population exhibiting G1=B1. In *A. flavus* populations, there may be fewer AF- isolates and more AF producers. And if, by some chance, *A. flavus* and *A. parasiticus* are capable of mating, then there is the potential for even more chemotype diversity in a field.

5. Current research into *A. flavus* and *A. parasiticus* populations

5.1 Is recombination a global phenomenon?

Investigations are underway that compare global populations of AF+ and AF- Aspergilli. The main goal is to ascertain whether or not recombination observed in the previously examined Georgia populations is a global phenomenon. Other insights of interest relate to the rates of recombination in geographically-isolated fields, and if the inferred recombination breakpoints are conserved among species across the globe. Finally, research is underway to determine if the factors that influence chemotype diversity are the same no matter where the population is found.

5.2 What influences chemotype diversity?

Now that recombination has been shown for representative *A. flavus* and *A. parasiticus* populations in the United States, with the potential for recombination to exist for global populations of AF+ fungi, investigations should explore the factors that generate chemotype diversity in these populations. Is there a correlation between recombination and the different chemotype profiles in a sample population? Will a clonal population have lower overall aflatoxin concentrations compared to an actively recombining, more diverse population? By examining aflatoxin concentration and *MAT* designation for each individual, it may be possible to prove that not only does fungal sex increase population diversity, but it also increases overall aflatoxin production. And what of the potential for niche adaptation? Is there a connection between chemotype diversity and the environment in which the population is found? Ecological factors such as precipitation, soil type and temperature may be important to population diversity.

5.3 What are the boundaries to gene flow among populations?

If geographically isolated populations of AF+ fungi all have similar rates of recombination and aflatoxin chemotype diversity, does this offer evidence of gene flow? Future research should explore the barriers to gene flow among populations. Obvious factors such as species composition and geography are expected to prevent the mixing of genetic material. Would it then be feasible to seek a non-native biocontrol strain to prevent potential genetic exchange and recombination? One might expect global, geographically isolated populations of AF+ fungi to be evolving at different rates, sharing little recent evolutionary history with their distant relatives on the other side of the world. Continued interest in how these populations are evolving will be integral to control of AF+ fungi that may contaminate the global food supply.

6. Biocontrol of aflatoxin contamination using non-toxigenic *A. flavus* strains

6.1 Testing stability and longevity of current biocontrol strains

Research is being conducted that tests the stability of the AF- phenotype for the biocontrol strains when crossed with an AF+ strain. If mating and recombination occur, and any of the offspring are AF+, then we must re-evaluate how biocontrol strains are selected. It might be necessary to adopt a next-generation biocontrol strain that is more resistant to recombination in the field. Future research will also require exploration of the longevity of the current biocontrol strains. If we cannot find evidence of the persistence of the biocontrol strain in the field, does that mean the strain dies off, or does that mean

the strain has recombined with indigenous AF+ strains and the AF- phenotype has been lost?
Researchers have also begun to explore how a biocontrol strain moves/persists in nature by creating a GFP-labeled transformant. Future endeavors could include releasing this labeled strain into a field and conducting a time-course study for the fluorescent strain. If an AF+ strain fluoresces then there will be indisputable evidence that the current biocontrol strains are recombining with indigenous strains as a result of successful out-crossing. Laboratory studies might include testing the aggressiveness of GFP-labeled strains when competing with AF+ strains, particularly in relation to various ecological conditions such as light, soil, and temperature.

6.2 Mating-type distribution in fields to be treated

Another important consideration in the selection of future biocontrol strains is the distribution of *MAT* idiomorphs in the field to be treated. If there is a predominance of one mating-type allele over another then it makes intuitive sense to release a biocontrol strain that shares the same *MAT* as the census population. Inundating a field in which the indigenous population is *MAT1-2* with a *MAT1-1* biocontrol agent may increase the incidence of recombination.

6.3 Genomic content of potential biocontrol strain

As mentioned earlier, it may be more beneficial to utilize a biocontrol strain that belongs to a lineage on an evolutionary dead-end. There has been no report of evidence that an AF- isolate which lacks the AF cluster can acquire a functional cluster configuration during recombination; therefore, an isolate lacking the aflatoxin gene cluster might be more difficult to "repair" than a point mutation in a gene subject to frequent shuffling. Another possible mechanism involves meiotic silencing, whereby unpaired homologs during prophase I of meiosis are silenced due to chromosomal misalignment (Shiu, Raju et al. 2001; Smith, Woloshuk et al. 2007).

7. Conclusion

A. flavus populations comprise a diverse assemblage of strains that are adapted to agricultural fields. Unlike other aflatoxigenic *Aspergillus* species, a portion of *A. flavus* populations has lost the ability to produce AFs. There is evidence that gene loss in the AF gene cluster of AF- isolates is irreversible and that balancing selection maintains non-aflatoxigenicity and lineage-specific gene loss in *A. flavus* populations (Donner, Atehnkeng et al.; Chang, Ehrlich et al. 2006; Ehrlich, Montalbano et al. 2007; Moore, Singh et al. 2009). Such AF- strains are being used for biocontrol of crop contaminations. Concerns have arisen about the possibility that introduced AF- strains of *A. flavus* used for biocontrol could re-acquire the ability to make AFs (Moore, Singh et al. 2009). If the introduced non-aflatoxigenic strain is more aggressive than native AF+ strains, a recombinant might have both increased AF-producing ability and increased ability to invade the plant. These concerns are based on the findings that *A. flavus, A. parasiticus* and *A. nomius*, aflatoxigenic fungi long considered to be incapable of forming a sexual state, can be induced to mate by crossing isolates from different mating types (Horn, Moore et al. 2009; Horn, Ramirez-Prado et al. 2009; Horn, Moore et al. 2011). The importance of understanding population structure is integral to preventing aflatoxin contamination of an increasingly precious food supply.

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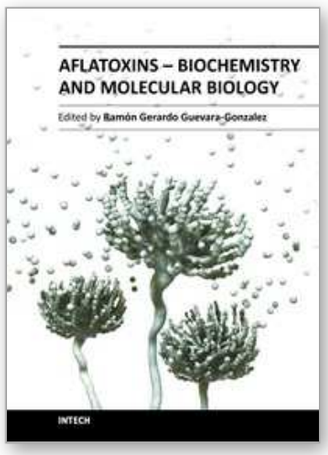

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Aflatoxins â€“ Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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