Biodiversity of *Aspergillus* section *Flavi* in the United States: A review

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Biodiversity of Aspergillus section Flavi in the United States: A review

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Abstract
Fungi belonging to Aspergillus section Flavi are of great economic importance in the United States due to their ability to produce toxic and carcinogenic aflatoxins in agricultural commodities. Development of control strategies against A. flavus and A. parasiticus, the major aflatoxin-producing species, is dependent upon a basic understanding of their diversity in agricultural ecosystems. This review summarizes our current knowledge of species and population diversity in the United States in relation to morphology, mycotoxin production and genetic characters. The high genetic diversity in populations of aflatoxigenic fungi is a reflection of their versatile habits in nature, which include saprotrophic colonization of plant debris in soil and parasitism of seeds and grain. Genetic variation within populations may originate from a cryptic sexual state. The advent of intensive monoculture agriculture not only increases population size but also may introduce positive selective pressure for aflatoxin production due to its link with pathogenicity in crops. Important goals in population research are to determine how section Flavi diversity in agricultural ecosystems is changing and to measure the direction of this evolution.

Keywords: Aflatoxin, Aspergillus flavus, Aspergillus parasiticus, corn, fungal diversity, groundnut, maize, peanut, plant parasitism, saprotrophy

Introduction
Fungi belonging to Aspergillus section Flavi are of great economic importance in the United States due primarily to the production of aflatoxins by several species in agricultural commodities. Aflatoxins are polyketide-derived furanocoumarins with known toxic, mutagenic, carcinogenic and immunosuppressive properties (Coulombe 1991; Turner et al. 2003). Outside of the United States, several aflatoxin outbreaks in humans, following consumption of contaminated grain, have been documented (Krishnamachari et al. 1975; Aziz-Baumgartner et al. 2005). Aflatoxin levels are strictly regulated in the United States and aflatoxins are very costly because of crop losses and activities associated with research and monitoring. In the southeastern United States alone, the cost of aflatoxins to the peanut industry averaged $26 million annually between 1993 and 1996 (Lamb and Sternitzke 2001). Our ability to control these fungi is dependent upon a basic understanding of their population biology, and biodiversity is a crucial component. Research involving biodiversity addresses important issues such as the origins of genetic diversity, the maintenance of diversity within populations and the effects of agriculture on diversity.

Aspergillus section Flavi species
A. flavus and A. parasiticus

The two dominant species from Aspergillus section Flavi in the United States, A. flavus and A. parasiticus, are responsible for the aflatoxin contamination of crops. A. flavus is widespread in agricultural regions where crops susceptible to aflatoxin contamination are grown, including corn in the South and Midwest, peanuts in the South, cottonseed in the desert Southwest, and figs and tree nuts in California (Payne 1998). A. flavus in the United States produces aflatoxins B₁ and B₂
A. flavus has been divided into two morphotypes based on sclerotium size: the L (large) strain, which produces sclerotia > 400 μm in diameter, and the S (small) strain, which produces numerous sclerotia < 400 μm in diameter in association with few conidial heads (Cotty 1989). However, sclerotium morphology is a poor indicator of phylogeny. A. flavus, as currently delimited, consists of several major lineages (Tran-Dinh et al. 1999; Kumeda et al. 2003) and DNA sequence analyses (Carbone et al. 2007) that are not yet fully defined because phylogenetic analyses often do not take into account recombination and balancing selection, both of which can introduce significant phylogenetic errors (Carbone et al. 2007). Geiser et al. (1998, 2000) divided A. flavus into groups I and II based on RFLPs of nuclear-coding genes and DNA sequences. Group I contains both L and S strains, whose aflatoxigenic representatives produce only B aflatoxins. Group II contains only S strains that produce B and G aflatoxins, or less commonly, only B aflatoxins. Only one group II strain was identified from the United States and that strain from Texas produced only B aflatoxins and no CPA. The B + G aflatoxin-producing S strains, which are present in Australia, Thailand, West Africa and Argentina (Saito and Tsuruta 1993; Cotty and Cardwell 1999; Geiser et al. 2000; Vaamonde et al. 2003), have not been reported from the United States (Cotty and Cardwell 1999; Horn and Dorner 1999). The S strain was originally described as A. flavus var. parvisclerotigenus, based on a type strain that produced only B aflatoxins (Saito and Tsuruta 1993). Frisvad et al. (2005) subsequently designated a neotype that produces B and G aflatoxins and raised the varietal rank to A. parvisclerotigenus.

A. parvisclerotigenus is also widespread in the United States but is generally less abundant than A. flavus. The species primarily infects peanuts and is uncommon in aerial crops such as corn and cottonseed (Horn 2005a). A. parasiticus has a lower temperature optimum for seed invasion than A. flavus, which might explain its preference for peanuts since pod soil temperatures are relatively cool compared to daytime temperatures of aerial crop fruits (Horn 2005b). A. parasiticus also appears to be more adapted to survival in the soil and less dependent on crop infection than A. flavus, as suggested by the sizable populations in agricultural soils north of where peanuts are cultivated (Angle et al. 1982; Horn and Dorner 1998). The species produces both B and G aflatoxins at generally high concentrations and non-aflatoxigenic isolates are uncommon; CPA is not produced (Horn et al. 1996). Non-aflatoxigenic isolates of A. parasiticus from the United States have been shown instead to accumulate O-methylsterigmatocystin (OMST), an immediate precursor to aflatoxin B₁ (Horn et al. 1996). A. parasiticus consists of several major lineages based on RAPD and heteroduplex panel analyses (Tran-Dinh et al. 1999; Kumeda et al. 2003) and DNA sequence analyses (Carbone et al. 2007), but the groups are not readily distinguishable morphologically.

Other species

Section Flavi species, other than A. flavus and A. parasiticus, that occur in the United States are mostly of minor importance to agriculture. A. nomius produces B and G aflatoxins and is often reported from dead or diseased insects (Peterson et al. 2001). The species has been isolated from diverse regions of the United States and is considered rare, possibly because it is easily confused with the morphologically similar A. flavus (Kurtzman et al. 1987). A. nomius populations are most reliably found in agricultural soils from the Mississippi Delta region and on parasitized alkali bees in Wyoming (Horn and Dorner 1998; Peterson et al. 2001). Another species, A. caelatus (Horn 1997), is widespread in soils from the southern United States and also has been reported in insect-damaged peanuts (Horn and Greene 1995; Horn and Dorner 1998). A. caelatus does not produce any major mycotoxins (Horn et al. 1996). A. tamarii is closely related to A. caelatus (Peterson et al. 2000) but consistently produces CPA (Dorner 1983; Horn et al. 1996). A. alliaceus, the only species from section Flavi with a known sexual state (teleomorph = Petromyces alliaceus), produces ochratoxin A (Bayman et al. 2002). Both A. tamarii and A. alliaceus are isolated commonly from soil and occasionally from crops, but their distributions within the United States are not well defined. Finally, two rare species from the United States include A. leporis, which is restricted to soil and rabbit dung in Wyoming and neighboring states, and A. avenaceus, which has been reported from soil in California (Christensen 1981).
Population dynamics

An examination of biodiversity in *Aspergillus* section *Flavi*, whether at the species or population level, requires an understanding of the life cycle of these fungi in agricultural ecosystems. *A. flavus* and *A. parasiticus* are metabolically versatile in their capacity to utilize many different substrates of plant, animal and man-made origin (Raper and Fennell 1965; Hasan 1999). Dead and living plant material comprises the dominant food source in nature for *A. flavus* and *A. parasiticus*. A wide range of plant-related organic compounds are degraded and utilized, including cellulose, pectin, lignin, tannins, cutin, starch, lipids and proteins (Olutiola 1976; Betts and Dart 1989; Guo et al. 1996; Long et al. 1998; Brown et al. 2001; Mellon and Cotty 2004; Batra and Saxena 2005). This ability to produce a large array of degrading enzymes is indicative of the opportunistic lifestyle of these fungi in which temporarily available resources are readily exploited (St. Leger et al. 1997).

**Saprotrophic life cycle in soil**

The life cycle of *A. flavus* and *A. parasiticus* in agricultural fields (Figure 1) can be divided into two stages: (1) colonization of plant debris in soil and (2) invasion of seeds and grain in actively growing crop plants. Most research has concentrated on invasion of crops due to economic and health concerns over aflatoxin contamination and, as a consequence, little is known about the saprotrophic activities of these fungi in soil. The ubiquity of *A. flavus* and *A. parasiticus* in soil has led to the conclusion that soil is a primary habitat for these fungi (Payne 1998; Horn 2005a). Soil densities of *A. flavus* in the United States can be very high in agricultural fields (>3000 CFU g⁻¹), particularly in regions where crops are routinely contaminated with aflatoxins (Horn et al. 1995; Orum et al. 1997). However, standard techniques for quantifying soil populations, such as dilution plating, tend to favor quiescent structures (especially conidia) and give little information on the actively growing component of populations in soil (Klich et al. 1992). Griffin et al. (1975) showed that many of the *A. flavus* colonies on soil dilution plates do, in fact, arise from conidia. Conidia remain dormant in soil due to soil fungistasis and germinate only in response to the appearance of a readily utilizable nutrient source (Griffin 1972; Hale and Griffin 1976). Colonization of plant debris *de novo* in soil, as opposed to the introduction of previously colonized plant material from crops, is supported experimentally by very few studies. Griffin and Garren (1976) observed an increase in soil populations of *A. flavus* following addition to soil of green rye manure from a cover crop, which would not be expected to initially harbor *A. flavus*. Root segments of various crops when buried in non-sterile soil were colonized by *A. flavus* when hyphae, conidia and sclerotia were used as a source of inoculum (Stack and Pettit 1984). In summary, the saprotrophic life cycle of section *Flavi* species

![Image of life cycle](image.png)
Infection of crops by primary inoculum in soil

Although the precise origins of populations of *A. flavus* and *A. parasiticus* in soil are unclear, extensive research supports the fact that these soil populations serve as an overwintering source of primary inoculum for infection of new crops (Figure 1). Peanuts, with their subterranean fruiting habit, are in direct contact with soil populations and do not rely on propagule dispersal for infection (Horn 2006). In contrast, aerial fruits of corn, cotton, and tree nuts are physically separated from primary inoculum and, therefore, infection of these crops requires dispersal by wind and arthropods to the site of infection. Drought stress and elevated temperatures promote infection by *A. flavus* and *A. parasiticus* in crops through a combination of reduced plant resistance and increased insect damage (Payne 1998).

Corn has been studied most intensively with respect to infection by primary inoculum in soil. The routes of corn infection by *A. flavus* are complex and include colonization of silks as well as woundng of kernels by insects (Widstrom 1979; Marsh and Payne 1984). Olanya et al. (1997) showed that waste corn kernels deposited on the soil surface create distinct gradients of both air-borne conidia and *A. flavus*-infested nitidulid beetles (Nitidulidae) away from the point sources of inoculum. Nitidulids are major vectors that carry *A. flavus* from crop debris in soil to developing corn ears (Lussenhop and Wicklow 1990). Colonized corn cobs that over-winter following harvest also serve as an important source of infection for both corn and cotton (Jaime-Garcia and Cotty 2004). The infection of cotton bolls by *A. flavus* is deposited onto soil. Soil population densities of *A. flavus* are released into the air and colonized plant material (Hill et al. 1984; Horn et al. 2004). Corn in the southern United States is typically harvested in August (Wilson et al. 1979; Bruns and Abbas 2004), which shortly follows the period (July) when corn ears in the Midwest are at the silk stage of development (National Agricultural Statistics Service; Iowa and Illinois, 2002–2006). Since corn ears are susceptible to *A. flavus* invasion for up to 30 days after silking (Jones et al. 1980; Widstrom et al. 1981), infection by conidia dispersed from long distances is possible. Such sources of infection relative to local primary inoculum in soil may be of minor importance, but likely introduce genetic diversity into *A. flavus* populations.

Crop effects on soil populations

Crops infected with aflatoxigenic fungi are the main source for replenishment of soil populations (Figure 1), especially at harvest when conidia are released into the air and colonized plant material is deposited onto soil. Soil population densities of *A. flavus* increase markedly following mechanical harvesting of infected corn and peanuts (Wicklow et al. 1984; Horn et al. 1995). Furthermore,
colonization of the crop material before harvest would be expected to give these fungi a competitive advantage over other microorganisms in soil.

Two lines of evidence suggest the importance of crops for the maintenance of soil populations. First, extremely low populations of section Flavi species are present in soils from virgin prairies, forests and fallow land that are in close proximity to cultivated fields with sizeable populations (Angle et al. 1982; Horn 2006). Pettit et al. (1973) indicated that A. flavus first appeared in soil from newly cultivated land during the second year of peanut cultivation. Second, soil population densities in the United States appear to be positively correlated with the frequency of drought in aflatoxin-susceptible crops (Horn and Dorner 1998). In the southern United States where droughts are common and winters are mild, densities remain high due to frequent replenishment by infected crops (Horn et al. 1995). Farther north where drought is infrequent, the cyclic relationship between crop infection and soil density is more pronounced. Shearer et al. (1992) showed high soil densities of A. flavus (1231 CFU g−1) in Iowa following corn harvest during the aflatoxin outbreak year of 1988. With no subsequent drought in following years, populations progressively decreased to 396 CFU g−1 in 1990 and finally to 0.3 CFU g−1 in 1993 (McGee et al. 1996).

**Diversity within populations**

**Colony morphology**

Populations of section Flavi species are diverse and comprise individuals that differ greatly in phenotype, including characters such as conidial color, sclerotium production, presence of diffusible pigments and growth rate (Raper and Fennell 1965; Christensen 1981; Horn et al. 1996). The morphological variation within populations can often be categorized according to vegetative compatibility groups (VCGs), defined as subpopulations whose members are genetically capable of forming stable hyphal anastomoses. For example, Horn et al. (1996) showed that variation in conidial color within populations of A. tamarii and A. caelatus, as measured by digital color-image processing, could be attributed to differences among VCGs. In the same study, A. parasiticus VCG 1, which is prevalent in peanut-growing regions of the United States (Horn and Dorner 1998), was recognized by its distinctive floccose growth habit, and VCGs of both A. flavus and A. parasiticus were distinguished according to sclerotium density, size and shape.

Morphology of these fungi shows considerable plasticity when influenced by environmental factors. The color of conidia, a character critical for differentiation of species within section Flavi, is sensitive to medium composition and age of culture (Raper and Fennell 1965). Sclerotium production is inversely related to sporulation, and highly sclerotial individuals generally sporulate poorly (Horn et al. 1996). The proportionality of sporulation and sclerotium production is influenced by light, nutrition and other environmental factors (Bennett et al. 1978; Calvo et al. 1999).

**Mycoxin production**

Research on mycoxin production within section Flavi populations has concentrated primarily on southern regions of the United States. In A. flavus populations from single fields in Georgia and Arizona, production of aflatoxin B1 among strains was extremely diverse, ranging from not detectable to very high (Bayman and Cotty 1993; Horn et al. 1996). Horn and Dorner (1998) also examined aflatoxin and CPA production in A. flavus soil populations along a 3300-km transect across the southern United States. A. flavus L-strain isolates (n = 774) that produced both aflatoxin B1 and CPA were most common (71%), followed by nonproducers of both mycotoxins (16%), CPA-only producers (12%), and aflatoxin B1-only producers (<1%). Most S-strain isolates (n = 309) produced both aflatoxin B1 and CPA (99%) and ≤1% produced CPA only or aflatoxin B1 only; no nontoxigenic S-strain isolates were detected. The L strain, on average, was less aflatoxigenic than the S strain. None of the L-strain isolates produced >300 μg ml−1 aflatoxin B1 whereas 26% of the S-strain isolates fell into this category. Other studies also have shown higher aflatoxin production by the S strain compared to the L strain (Cotty 1989, 1997). A. parasiticus generally produces high levels of aflatoxins and, therefore, populations are considerably less diverse in aflatoxin production than those of A. flavus L strain (Horn et al. 1996).

As with morphological characters, variation in aflatoxin production by A. flavus and A. parasiticus and in CPA production by A. flavus can be categorized according to VCGs, with little variation occurring within a VCG and most of the variation occurring among VCGs (Horn et al. 1996). The mechanisms underlying variation in aflatoxin production have been examined to some extent at the molecular level. The inability of A. flavus to produce G aflatoxins can be attributed to the absence of portions of aflU (=cypA) and aflF (=norB) genes in the aflatoxin gene cluster required...
for G aflatoxin synthesis (Ehrlich et al. 2004). Many A. flavus strains that do not produce aflatoxins contain deletions of various sizes in the gene cluster (Chang et al. 2005) or exhibit a single nucleotide substitution in the aflC (=pksc) gene (Ehrlich and Cotty 2004). Those A. parasiticus strains that do not produce aflatoxins instead accumulate OMST due to several point mutations on the aflQ (=ordA) gene (Yu et al. 1998). The genetic factors resulting in different degrees of aflatoxin production among aflatoxigenic strains are not understood. Differential responses to environmental cues affecting aflatoxin stimulation/repression, such as carbon and nitrogen sources and pH, may account for some of the differences among strains (Payne and Brown 1998).

Genetic characters

Several techniques have been used in recent years to describe the genetic variation in section Flavi populations. Vegetative compatibility reactions in the delimitation of VCGs for population analyses (Bayman and Cotty 1991; Horn and Greene 1995) provide a multilocus phenotype for measuring genetic diversity. The formation of stable hyphal fusions is under the control of a series of het loci whose alleles must all be identical (Leslie 1993). A. nidulans contains a minimum of eight het loci distributed over five linkage groups (Anwar et al. 1993). The validity of vegetative compatibility as a tool for measuring genetic diversity was indicated by two blind studies in which populations of A. flavus and A. caelatus, which had been previously characterized according to VCGs, were examined using DNA fingerprinting (McAlpin et al. 2002, 2005). In both species, populations showed a nearly identical correspondence between VCGs and DNA fingerprint groups. DNA fingerprinting has also been used to describe the high diversity of A. flavus and A. parasiticus populations in an Illinois corn field (McAlpin et al. 1998; Wicklow et al. 1998). Other molecular techniques, such as DNA sequencing, which were previously limited by the sizable numbers of fungal isolates necessary for population analyses, are now possible due to the lower cost of equipment and increased automation. Carbone et al. (2007) examined an A. parasiticus population from Georgia that had been earlier subdivided into VCGs and characterized according to morphology and mycotoxin production (Horn & Greene 1995; Horn et al. 1996). Intergenic regions separating 21 genes in the aflatoxin gene cluster were sequenced, since they harbor a high degree of variation and are presumably neutrally evolving and thus can serve as a molecular clock. Ten haplotypes (alleles) were present among the 17 VCGs. Individual VCGs were not divided according to haplotype, but in many instances VCGs were grouped into single haplotypes because VCGs had formed after establishment of the haplotypes during the evolutionary history of the species.

Origins of genetic variation

The high genetic diversity in A. flavus and A. parasiticus populations has long intrigued researchers because the two species have no known sexual stage and are presumably strictly mitotic in reproduction. Genetic variation in fungi ultimately arises from mutation. Carbone et al. (2007) showed that three A. parasiticus haplotypes (H7, H8, H10) were distinguished from one another by 1–2 mutations within the intergenic regions of the aflatoxin gene cluster and had originated less than one million years ago. Another haplotype (H2) had accumulated at least 94 mutations within the gene cluster since diverging from its most recent common ancestor 5–10 million years ago. These mutation numbers represent the amount of variation over time expected for a neutrally evolving genomic region. However, this same study clearly demonstrated that only a series of recombination events overlying the mutations could account for the complex phylogeny within A. parasiticus.

Parasexuality is one means by which recombination can occur in asexual fungi. The parasexual cycle involves fusion of genetically different nuclei within a heterokaryon to form a diploid nucleus which, through a series of mitoses involving crossing over and chromosome loss, eventually returns to a haploid state that differs genetically from the parental nuclei (Pontecorvo 1956). Parasexuality has been demonstrated in both A. flavus and A. parasiticus under laboratory conditions (Papa 1973, 1978). There is some evidence for parasexuality in nature in the rice blast fungus (Magnaporthe grisea) (Zeigler et al. 1997), but this has not been demonstrated in section Flavi species. Several lines of evidence argue against the importance of parasexuality in recombination within section Flavi populations. First, heterokaryosis involving the presence of genetically different nuclei in a single fungal cell is a prerequisite for parasexuality. A major constraint for heterokaryon formation, therefore, is vegetative compatibility in which stable hyphal fusions can occur only between individuals belonging to the same VCG. The high VCG diversity within A. flavus and A. parasiticus populations greatly reduces the probability of contact between vegetatively compatible individuals. Second, individuals within a VCG are probably predominantly clonal, which would limit the potential for heterokaryosis, though some
evidence suggests that a low degree of genetic diversity occurs within VCGs (Bayman and Cotty 1993; McAlpin et al. 2002). Finally, Aspergillus heterokaryons are often unstable and tend to segregate into their component nuclei (Ishitani and Sakaguchi 1956).

Recombination may also be indicative of sexuality in section Flavi species. Geiser et al. (1998) reported evidence for cryptic recombination in A. flavus group I based on a lack of concordance among different gene genealogies. Similar conclusions were obtained for A. nomius owing to the noncongruence of gene trees for four genes (Peterson et al. 2001). Carbone et al. (2007) reported that the aflatoxin gene cluster in A. parasiticus shows significant linkage disequilibrium over the evolutionary history of the species and that the cluster can be divided into five distinct recombination blocks. The presence of mating-type genes in section Flavi species suggests that sexuality may account for cryptic recombination. A. oryzae, a domesticated form of A. flavus (Kurtzman et al. 1986), has separate mating-type genes MAT1-1 and MAT1-2 in individuals, indicating that the species is heterothallic (Paoletti et al. 2005). Heterothallism has also been demonstrated in A. parasiticus whose populations contain both mating types (Carbone et al., 2007). It is not known whether genes associated with sexuality in these examples are currently functional. Evidence of recombination in A. parasiticus within the last one million years (Carbone et al. 2007) suggests that sexuality may still be occurring at a low rate in natural populations. Section Flavi species, such as A. flavus, A. parasiticus, A. nomius and A. caelatus, often produce sclerotia that are structurally similar to immature ascostromata of the homothallic species A. alliaceus, which infrequently produces ascospores after extended periods of incubation (McAlpin and Wicklow 2005). Sclerotia may, therefore, serve as ascostromata under specific environmental conditions when opposite mating types are in close proximity. Dyer and Paoletti (2005) have proposed that A. fumigatus, another heterothallic species with cryptic recombination, has evolved into a weakly fertile and predominantly asexual species whose rare ability to mate is sufficient to maintain diversity within populations.

Factors influencing biodiversity

Agriculture

Complex processes are influencing population structure of section Flavi species in agricultural fields in the United States, as evidenced by the high genetic diversity within populations (Bayman and Cotty 1991; Horn and Greene 1995; McAlpin et al. 1998; Wicklow et al. 1998), the rapid fluxes in population composition (Bayman and Cotty 1991; Orum et al. 1999), the distinctive spatial patterns of populations in soil (Orum et al. 1997, 1999; Jaime-Garcia and Cotty 2006) and the marked differences in aflatoxigenicity according to geography (Horn and Dorner 1999). Inherent in this complexity is the influence of agriculture on genetic diversity within populations of these fungi. Section Flavi populations with high genetic diversity in agricultural habitats are often assumed to have arisen from natural populations (Carter et al. 1998). Comparisons between populations from native habitats and those from agricultural fields in the same geographic area are necessary to detect any influence of agriculture on genetic diversity, assuming that extensive migration from fields to native habitats does not preclude such studies. Alternatively, changes in population diversity could be monitored over many crop seasons when a natural habitat is converted to agriculture. The only in-depth study of natural populations of section Flavi species was conducted in the Sonoran Desert of southern Arizona where the collecting location was far removed from agriculture (Boyd and Cotty 2001).

Extensive cultivation of monoculture crops might be expected to lower genetic diversity in A. flavus and A. parasiticus populations, especially if there is positive selection for pathogenicity in invading crops (Burdon and Silk 1997). Similarly, field soil populations that serve as a primary source for crop infection might also have reduced diversity due to the disruption of natural ecological niches through tillage and low plant diversity. Despite these influences, populations from crop plants and field soils remain highly diverse genetically. Widespread dispersal of conidia may account for the rapid temporal shifts in VCG and S-strain composition of populations (Bayman and Cotty 1991; Orum et al. 1999). Migration between populations typically increases genetic diversity (Burdon and Silk 1997) and would theoretically expand the availability of different ecological niches beyond the boundaries of a single field. Furthermore, selective pressures undoubtedly differ between the two major stages in the life cycle of section Flavi species: (1) the saprotrophic colonization of organic matter in soil in which competition with other microorganisms is intense and (2) the parasitic invasion of seed and grain crops in which overcoming defensive host structures and chemicals is critical (Figure 1). Populations at each stage, and the selective pressures acting upon them, are not clearly separable, since soil populations serve as the primary source for plant infection and infected plant parts contribute largely to soil populations. The contribution of a purely saprotrophic life cycle to soil populations is not
known, but its presence could further confound population analyses if the genetic composition differs from that associated with crops.

Selection under experimental conditions

Laboratory studies have provided some insight into evolutionary processes shaping *A. flavus* populations when under intense selective pressure for a relatively small number of generations. *A. flavus* strains originating from different substrates in nature initially show little evidence of host specialization (St. Leger et al. 2000), but the species is highly adaptable when repeatedly subcultured on specific substrates. For example, only five generations of serial infections of insect larvae were necessary to show phenotypic changes in *A. flavus* when growing on the host (increase in sporulation and shorter time interval in appearance on cadavers) and a concomitant decrease in ability to grow as a saprotroph on an agar medium (Scully and Bidochka 2005). The genetic diversity as measured by amplified fragment length polymorphism (AFLP) fingerprints also decreased during serial larval infections, possibly due to a genetic bottleneck effect, but remained the same or increased on the agar medium in which selective pressure was minimal (Scully and Bidochka 2006a); one strain emerged from the serial infections that was dependent upon insect parasitism for sporulation (Scully and Bidochka 2006b).

Another example is illustrated by *A. oryzae*, a domesticated fungus that evolved relatively recently from *A. flavus* through repeated subculturing on cereals for production of koji used in food fermentation (Wicklow 1984a). Conidia of *A. oryzae* are larger and germinate faster than those of *A. flavus*, an adaptation for effectively competing with other microorganisms on cereal substrates (Wicklow 1984b). Subculturing of aflatoxigenic *A. flavus* on an enriched agar medium for as few as 20 generations often resulted in an *A. oryzae*-like phenotype that included loss of aflatoxin production, reduced sporulation and sclerotium formation, floccose growth and brown conidial color (Horn and Dorner 2002). This same study showed that aflatoxin production and formation of reproductive structures tend to be maintained over successive generations by adverse environmental conditions, such as high temperature, low pH and low nutrients, but not by competition with yeast and filamentous fungi. Thus, *A. oryzae* and *A. flavus* exhibit phenotypes that are adaptive to their respective koji and natural environments (Wicklow 1984a). Experimental studies have not been conducted to address the question as to whether seed and grain crops in agricultural ecosystems also have selective influences on section *Flavi* populations.

Balancing selection in maintaining chemotypes

Recent molecular work has provided some insight into evolutionary processes with respect to the aflatoxin gene cluster. Ehrlich et al. (2005) have estimated that in section *Flavi*, gene order, intergenic distances and binding sites of the master transcriptional regulator gene aflR have been conserved for at least 25 million years, suggesting strong purifying selection for maintaining the aflatoxin gene cluster. Selective processes are also responsible for the signature chemotypes that are diagnostic of species within section *Flavi*, such as G aflatoxin production by *A. parasiticus*, *A. nomius* and an unnamed taxon (Ehrlich et al. 2005; Carbone et al. 2007). Especially intriguing is evidence of balancing selection resulting in the persistence of different chemotypes within populations of each species (Carbone et al. 2007). Examples of chemotypes in *A. parasiticus* that differ from the typical B + G aflatoxin-producers include strains that are non-toxigenic (Carter et al. 2002), strains that accumulate only OMST (Horn et al. 1996) and strains that produce different ratios of G₁ to B₁ (Horn et al. 1996). Examples of chemotypes in *A. flavus* L strain that differ from the typical B-aflatoxin producers include strains that are non-aflatoxigenic due to various deletions and substitutions in the aflatoxin gene cluster (Ehrlich and Cotty 2004; Chang et al. 2005) and strains that produce exclusively aflatoxin B₂ (Schroeder and Carlton 1973). The factors responsible for balancing selection in the maintenance of different chemotypes within populations are not understood, but adaptations to different ecological niches may play a role (Carbone et al. 2007). Furthermore, selection may act upon aflatoxin pathway metabolites in conjunction with other secondary metabolites. For example, Dowd (1988) showed a synergistic effect on aflatoxin B₁ toxicity toward several species of insects in the presence of kojic acid.

Aflatoxins

Production of aflatoxins by *Aspergillus* species involves approximately 25 genes in a complex biosynthetic pathway that requires considerable expenditure of energy (Yu et al. 2004). As stated earlier, there is strong purifying selection for maintaining the aflatoxin gene cluster in section *Flavi* (Ehrlich et al. 2005); therefore, aflatoxins are presumed to be advantageous to the fungus in some manner. Aflatoxins have toxic effects on a wide range of organisms (Eaton and Groopman 1994).
Current knowledge does not permit the separation of toxic effects that are simply fortuitous from those that are of benefit to the fungus. Many of the theories concerning the role of aflatoxins have merit, but few adequately address the observation that aflatoxigenic and non-aflatoxigenic strains successfully coexist in nature. It has been argued that if aflatoxins are advantageous to the fungus, then aflatoxin producers should eventually displace non-producers over evolutionary time (Carter et al. 2002). Instead, populations are highly diverse in their capacity to produce aflatoxins.

**Competition**

The toxicity of aflatoxins has led to suggestions that they have a role in competition, either as antibiotics against microorganisms or as toxic agents/feeding deterrents against arthropods. Aflatoxins show no or low toxicity against bacteria (Arai et al. 1967), and inhibition of fungal sporulation varies considerably according to species and growth medium (Reiss 1971). When added to soil, aflatoxins have minimal inhibitory effects on microbial growth, possibly due to their rapid degradation by microorganisms (Angle and Wagner 1980, 1981).

Toxicity of aflatoxins toward arthropods, which often compete with fungi for seeds and grain (Wicklow 1988), is more pronounced. Aflatoxins have toxic and chemosterilizing effects on a number of insect pests of crops (Moore et al. 1978; McMillian et al. 1980). Despite these properties, aflatoxins are sometimes ineffective in protecting the fungus and its substrate from insect feeding. Larvae beyond the first instar of development are often insensitive to aflatoxins and lengthy feeding periods are often required before toxic effects are expressed (Jarvis et al. 1984). Aflatoxins may be more effective against arthropods when part of a suite of secondary metabolites produced by the fungus (Dowd 1988; Wicklow and Dowd 1989), particularly when present in resistant structures such as sclerotia (Wicklow et al. 1994). Insects are also occasionally parasitized by section *Flavi* species, especially *A. nomius* and *A. bombycis* (Peterson et al. 2001). Aflatoxins have been reported as non-essential to insect parasitism (Drummond and Pinnock 1990), but further research is necessary to confirm this.

**Fungal morphogenesis**

The aflatoxin pathway has been linked to morphological development in section *Flavi* species. Aflatoxin production and sporulation exhibit similar responses to growth conditions and metabolic inhibitors, and aflatoxin mutants are usually aberrant in their ability to sporulate (Kale et al. 1996). Wilkinson et al. (2004) demonstrated that in *A. nidulans*, a species that produces the aflatoxin pathway intermediate sterigmatocystin, there is an increase in sporulation with each progressive step in the pathway. Aflatoxin/sterigmatocystin production and sporulation both appear to be regulated by a signal transduction pathway (Calvo et al. 2002; Roze et al. 2004). The aflatoxin pathway and sclerotium development have also been linked. Sclerotium production decreases with accumulation of aflatoxin pathway intermediates and is often accompanied by changes in sclerotium morphology (Trail et al. 1995; Chang et al. 2001). Since reproductive structures, such as conidia and sclerotia, are strongly acted upon by natural selection, there may be indirect selective effects on aflatoxins through their link with developmental processes.

**Metabolic functions**

Recent research has implicated aflatoxin biosynthesis in the functioning of several metabolic processes. One possible function is the neutralization of reactive oxygen species, which form during plant infection and under environmental stress, by the oxygenated polyketides associated with the aflatoxin pathway (Jayashree and Subramaniam 2000; Kim et al. 2005). The addition of oxidants, such as hydrogen peroxide, to culture media increases aflatoxin production (Narasaiah et al. 2006), whereas various phenolics acting as strong antioxidants suppress aflatoxin biosynthesis (Kim et al. 2004). The mechanism by which aflatoxin biosynthesis alleviates oxidative stress has not been elucidated. Another link between aflatoxins and metabolic functions is suggested by Price et al. (2006), in which the *aflR* gene was shown to not only regulate genes in the aflatoxin gene cluster but also several other genes associated with sugar utilization and nitrogen metabolism.

**Plant pathogenesis**

Mycotoxins have long been suspected of being involved in plant pathogenesis (Desjardins and Hohn 1997), and aflatoxins are no exception. The arguments against the role of aflatoxins in crop invasion are primarily based on: (1) the low toxic effects on plants and (2) the capacity of non-aflatoxigenic *A. flavus* strains to infect plants. Research on the toxicity of aflatoxins toward plants has concentrated on seed germination and on growth of callus tissue, embryos and seedlings under laboratory conditions (Dashek and Llewellyn 1983; McLean et al. 1995). The toxicity of aflatoxins to maturing seeds and
A. flavus inoculations with high densities of non-aflatoxigenic strains, is supported by biological control, in which field inoculations with high densities of non-aflatoxigenic A. flavus and/or A. parasiticus reduce aflatoxin contamination by competitively excluding native aflatoxigenic strains in peanuts, cottonseed and corn (Dorner 2005). Effective biological control necessitates very high ratios of non-aflatoxigenic to aflatoxigenic strains, but when densities of biocontrol strains are the same as or only slightly higher than densities of aflatoxigenic strains in peanut fields, aflatoxin-producing strains preferentially invade peanuts (Dorner and Horn 2007).

The role of aflatoxins in crop infection was also suggested by Horn and Dorner (1999) in an examination of soil populations along a transect across the United States. Greater than 95% of A. flavus L strains in the southern peanut-growing region of Georgia/Alabama produced aflatoxins, as opposed to approximately 50% in the northernmost peanut-growing region of North Carolina/Virginia. Since crop infection is more prevalent farther south, those populations might reflect a higher selective pressure for producing aflatoxins. Coty (1997) observed a similar latitudinal effect on aflatoxin-producing potential of populations in the southern United States and, in the Midwest where drought and A. flavus outbreaks are infrequent, non-aflatoxigenic strains are common (Horn 2005a). Aflatoxicogenic of populations also appears to vary according to the type of crop. Schroeder and Boller (1973) reported that in Texas, 96% of A. flavus isolates from peanuts produced aflatoxins, in contrast to lower percentages in cottonseed (79%), sorghum (49%) and rice (35%). Understanding the role of aflatoxins in crop infection requires detailed physiological studies using crop seeds and grain, as well as extensive population analyses to discern any positive selection for aflatoxin production in invasion of crops.

Conclusions

Modern molecular techniques are redefining our ideas concerning the origins of genetic diversity within Aspergillus section Flavi, as well as the structure and evolutionary history of populations. Genetic diversity of these fungi must be examined in the context of current practices of intensive agriculture, which arose relatively recently in the United States (<200 years ago). Peanuts, for example, have been grown on a small scale since settlement but rapid expansion of cultivation did not occur until 1860–1870 (Higgins 1951). Assuming that agricultural populations arose from local wild populations, then the rapid clonal expansion resulting in large population sizes and the selective pressures associated with monoculture crop infection might have profound effects on current and future levels of diversity. Therefore, important goals in population research are to determine how diversity is changing in section Flavi species and to measure the direction of this evolution.

References

and its possible role in infection of corn kernels. Phytopathology 86:824–829.


