Sequence comparison of \textit{aflR} from different \textit{Aspergillus} species provides evidence for variability in regulation of aflatoxin production

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Abstract

Aflatoxin contamination of foods and feeds is a world-wide agricultural problem. Aflatoxin production requires expression of the biosynthetic pathway regulatory gene, \textit{aflR}, which encodes a Cys$_6$Zn$_2$-type DNA-binding protein. Homologs of \textit{aflR} from \textit{Aspergillus nomius}, \textit{bombycis}, \textit{parasiticus}, \textit{flavus}, and \textit{pseudotamarii} were compared to investigate the molecular basis for variation among aflatoxin-producing taxa in the regulation of aflatoxin production. Variability was found in putative promoter consensus elements and coding region motifs, including motifs involved in developmental regulation (AbaA, BrlA), regulation of nitrogen source utilization (AreA), and pH regulation (PacC), and in coding region PEST domains. Some of these elements may affect expression of \textit{aflJ}, a gene divergently transcribed from \textit{aflR}, that also is required for aflatoxin accumulation. Comparisons of phylogenetic trees obtained with either aligned \textit{aflR} intergenic region sequence or coding region sequence and the observed divergence in regulatory features among the taxa provide evidence that regulatory signals for aflatoxin production evolved to respond to a variety of environmental stimuli under differential selective pressures. Phylogenetic analyses also suggest that isolates currently assigned to the \textit{A. flavus} morphotype S$_{BG}$ represent a distinct species and that \textit{A. nomius} is a diverse paraphyletic assemblage likely to contain several species.

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IDT: Aflatoxin; \textit{Aspergillus}; \textit{aflR}; \textit{aflJ}; Transcription regulation; Phylogenetics; PEST domains; PacC; AreA; BrlA

1. Introduction

\textit{Aspergillus} section \textit{Flavi} includes the species, \textit{A. parasiticus}, \textit{A. flavus}, \textit{A. nomius}, \textit{A. bombycis}, and \textit{A. pseudotamarii}, which under certain conditions produce highly toxic and carcinogenic aflatoxins (Cotty and Cardwell, 1999; Egel et al., 1994; Ito et al., 2001; Peterson et al., 2001). In addition, more than 50 other species of filamentous fungi, including several species of \textit{Penicillium} and a distantly related \textit{Chaetomium}, have been reported to synthesize sterigmatocystin and other aflatoxin precursors (Barnes et al., 1994; Frisvad, 1985). Crops can become contaminated with aflatoxins when conditions favor growth of these fungi (Cotty et al., 1994). Annual costs resulting from crop losses and the need to limit food contamination have been estimated to be more than $100 million (Robens, 2001). Of the aflatoxin-producing species, \textit{A. flavus} and \textit{A. parasiticus} are the most common species implicated as causal agents of aflatoxin contamination (Cotty et al., 1994). Roles of agriculture in structuring communities of aflatoxin-producing fungi are unclear (Bayman and Cotty, 1993). Aflatoxin-producing fungi are a mosaic of species that belong to divergent clades (Cotty et al., 1994). Within certain clades and along some lineages aflatoxin-producing ability is highly conserved, but in other clades aflatoxin production is either highly variable or lacking (Egel et al., 1994; Geiser et al., 2000). Aflatoxin-producing fungi reproduce in diverse ecological niches throughout warm climates (Cotty et al., 1994). Transcriptional regulation of aflatoxin biosynthesis might be contingent upon environmental signals particular to...
different niches. Nitrogen source, pH, and even antimi-
crobial agents are known to differentially influence
aflatoxin biosynthesis among species, isolates, and
strains (Cotty, 1988; Cotty and Cardwell, 1999). Vari-
ation in the molecular structure of aflatoxin regulatory
genes and the molecular basis for divergent regulation of
aflatoxin synthesis among strains has not been de-
scribed.

The 23 genes involved in aflatoxin biosynthesis in A.
flavus and A. parasiticus are part of a 75 kb cluster (Trail
et al., 1995; Yu et al., 1995). Most of the genes are co-
regulated by a single pathway-specific, DNA-binding
protein, AflR (Ehrlich et al., 1999b). The gene encoding
AflR resides in the cluster between early and late-acting
genes involved in aflatoxin biosynthesis. AflR has a
Cys6Zn2 DNA-binding domain and C-terminal tran-
scription activation domain typical of GAL4-type
fungal and yeast transcription factors. AflR binds to
the partially palindromic consensus sequence 5'-TCGN3
CGR-3' found in promoters of most of the aflatoxin
biosynthesis genes (Chang et al., 1995; Ehrlich et al.,
1999b).

A gene, aflJ, is divergently transcribed from aflR. This
gene encodes a protein, AflJ, which also appears to be
involved in aflatoxin gene regulation (Meyers et al.,
1998). Although an exact function for this protein has not
yet been identified, preliminary evidence suggests
that it modulates AflR activity (P.-K. Chang, unpub-
lished results). AflJ was found to be necessary for ac-
cumulation of some of the early precursor metabolites
involved in the aflatoxin biosynthetic pathway (Meyers
et al., 1998). In addition, transformants containing an
extra copy of aflR but lacking an extra copy of aflJ have
a reduced level of expression of aflR compared to
transformants containing a second copy of both genes
(Chang et al., 1995). In this report, we compared the
nucleotide sequences of the aflJ/aflR intergenic region
and aflR coding region in 28 isolates of five species of
aflatoxin-producing fungi within Aspergillus section
Flavi to deduce variations in regulatory region motifs
and inferred protein structure, as well as phylogenetic
relationships among these species.

2. Materials and methods

2.1. Fungal isolates

Twenty-eight fungal isolates belonging to Aspergillus
section Flavi and known to produce aflatoxins were used
in this study (Table 1). Isolates thought to represent
maximum divergence among aflatoxin producing species
were chosen. A. flavus isolates included morphotypes
which produce only B aflatoxins and either numerous
small sclerotia (average diameter < 400μm) or fewer,
larger sclerotia (Cotty, 1989). The former type has been
called the S strain of A. flavus (Cotty, 1989) or A. flavus
var. parvisclerotigenus (Saito and Tsuruta, 1993), while
the latter has been called the L strain of A. flavus (Cotty,
1989). Atypical A. flavus isolates which produce both B
and G aflatoxins were also included. These were de-
scribed previously as an unnamed taxon (Egel et al.,
1994; Hesseltine et al., 1970), the SBG strain of A. flavus
(Cotty and Cardwell, 1999), or A. flavus Group II
(Geiser et al., 2000; Geiser et al., 1998). A. nomius iso-
lates were chosen which show considerable morpho-
logical, physiological, and genetic divergence. Ex type
cultures of A. nomius (Kurtzman et al., 1987), A.
bombycis (Peterson et al., 2001), and A. pseudotamarii
(Ito et al., 2001) were selected but not ex type cultures of
A. flavus and A. parasiticus, because those isolates do
not produce detectable quantities of aflatoxins. A. par-
asiticus CP-461, which produces the penultimate pre-
cursor, O-methylsterigmatocystin (OMST), but not aflatoxin,
was included because of its importance in studies of
the molecular biology of aflatoxin production
(Bhatnagar et al., 1987). Active fungal cultures were
maintained without light on 5/2 agar (5% V-8 vegetable
juice, 2% agar, and pH 5.2) at 31 °C. Isolates were stored
at 4 °C as 3-mm plugs of sporulating culture (5–7-days-
old on 5/2 agar) in sterile distilled water. Aflatoxin
production was assessed as previously described after
growth of the fungi on a minimal medium of Adye and
Mateles (1964) with 22.5 mM urea instead of ammo-
nium sulfate as the sole nitrogen source (Cotty, 1997;
Cotty and Cardwell, 1999).

2.2. Sequence analysis

Genomic DNA and RNA from 3- to 5-day fungal
cultures was isolated (DNeasy and RNeasy Kits, Qia-
gen, Valencia, CA) from mycelia ground to a powder
in liquid nitrogen (Ehrlich et al., 1999a). Polymerase
chain reaction (PCR) on the genomic DNAs was done
using the oligonucleotides shown in Table 2. Primers 1
and 5 overlapped the translational start sites of the A.
parasiticus aflJ and aflR, respectively (Fig. 1) (Ehrlich
et al., 1999a). PCR of coding regions of A. flavus and A.
parasiticus aflRUs used primers 6 and 15, whereas
PCR of the other taxa used primers 7 and 16 which
overlapped the coding region for highly conserved
portions of the protein. Sense primers 2–4 in the aflJ/aflR
intergenic region were used with antisense primers
12–16 to complete the sequence at the 5'-end of
the aflR-coding region. 3'-RACE PCR (SMART RACE
cDNA amplification kit from Clonetech, Palo Alto,
CA) with primer 11 and A. nomius and A. pseudotamarii
DNAs was used to fill in the end of the 3'-coding
region that was not obtainable by the genomic DNA
amplifications. Other primers listed in Table 2 were
used to fill in and verify sequence at the ends of the
coding regions for some of the A. nomius, A. bombycis,
A. flavus, and A. parasiticus strains. PCR products were cloned using the TA cloning kit from Invitrogen (Carlsbad, CA), and dye terminator dideoxy sequencing was done by standard methods. All sequences were determined in both directions. Selected sequences were obtained on several independent clones and found to be identical. Sequences for the combined aflJ/aflR intergenic region and coding regions were deposited in GenBank as accession numbers AF441414-41. Detection of regions rich in PEST motifs in the inferred AflR protein sequence was accomplished with the PEST find algorithm (http://www.at.embnet/embnet/tools/bio/PESTfind/).

### Table 1

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<th>Other name</th>
<th>Species</th>
<th>Type</th>
<th>Location</th>
<th>Substrate</th>
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### 2.3. Alignments and phylogenetic analyses

DNA sequences were aligned using ClustalW in DNAMAN (Lynnon Biosoft, Vandreuil, Canada). Data sets included aligned DNA sequences from the aflJ/aflR intergenic region and the aflR coding region. Sequence alignments have been deposited at TreeBASE (http://www.treebase.org/treebase/) under accession number SN745, matrix accession number M1177. Phylogenetic trees were obtained by parsimony analyses using heuristic search methods with stepwise sequence addition and the tree-bisection-reconnection (TBR) branch-swapping algorithm (PAUP*ver4.0b10 Macin-
tosh, Sinauer Associates, Sunderland, MA). Node support was assessed with 1000 bootstrap replicates. Gaps were treated as missing. *A. bombycis* was chosen as the outgroup based on previously reported relationships among *Aspergillus* species (Peterson, 1997; Peterson et al., 2001). The partition homogeneity test (PHT) in PAUP* (Farris et al., 1994; Swofford, 1998) was performed on parsimony informative sites only, with 1000 randomized data sets using heuristic search methods with stepwise sequence addition. A two-tailed Kishino–Hasegawa (KH) test using 1000 RELL bootstrap replicates (Kishino and Hasegawa, 1989) in PAUP* was employed to further assess the likelihood of the different tree topologies.

### 3. Results

#### 3.1. Aflatoxin production

*A. flavus* L and S₈ and *A. pseudotamarii* isolates produced B aflatoxins only, whereas all of the other

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**Table 2**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
<th>Direction</th>
<th>Region</th>
<th>Homology</th>
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<td>AF/AP</td>
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*R* oligonucleotide primer numbers correspond with the numbers shown in Fig. 1.

**Sequences are written 5’ to 3’; k = G or T, s = C or G.**

**Numbering is from the beginning of the aflJ ORF.**

**s = sense, as = antisense; orientation with respect to the aflR ORF.**

**JR intergenic = the non-transcribed region between the translational start sites for aflJ and aflR; Zn cluster is the region encoding the Zn₂Cys₆ binuclear cluster DNA binding domain of AflR; R-coding = the coding region for aflR. The 3’-noncoding region is the region encompassing the translational termination signal and the polyadenylation site.**

**AF = A. flavus, AP = A. parasiticus, AB = A. bombycis, AN = A. nomius, APT = A. pseudotamarii.**

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Fig. 1. Schematic diagram of the aflR/aflJ intergenic and aflR coding regions. The gene aflJ is divergently transcribed from aflR as indicated by the horizontal arrow. Shaded rectangles indicate the locations of putative coding region domains in the DNA. G1 to G5 shown below the aflJ/aflR intergenic region indicate HGATAR sites (putative AreA-binding sites). Other putative transcription factor binding sites are shown above the intergenic region sequence. Numbers in open arrows indicate the location and direction of oligonucleotides used for PCR (Table 2).
aflatoxin-producing fungi produced both B and G aflatoxins (Table 1). On medium containing urea as the nitrogen source, aflatoxin production by the A. flavus S_B and S_BG isolates, the A. parasiticus isolates, and the seven typical A. nomius isolates (N13–15; 20–23) was consistently higher and less variable than that of the A. flavus L isolates, the A. bombycis isolates, the genetically divergent A. nomius (N16) (Engel et al., 1994), and the three A. nomius O isolates (N17–19) (Table 1). Aflatoxin production varied 4000-fold between the two A. bombycis isolates, and 1000-fold among the four A. flavus L isolates. A. pseudotamarii isolates produced approximately 3-fold lower levels of aflatoxin than did A. flavus S_B isolates.

### 3.2. aflR Sequence comparison

Comparison of aflR promoter and coding region sequences from 28 isolates of five aflatoxin-producing Aspergillus species showed differences in length and homology. The aflR/aflJ intergenic region ranged from 689 bp in A. pseudotamarii to 745 bp in A. flavus S_BG isolates (Table 3), while the coding region varied from 1305 in A. flavus S_BG to 1350 bp in A. pseudotamarii isolates. The aflR/aflJ intergenic region of A. parasiticus was 98, 98, 92, 80, 75, and 78% identical to those of A. flavus L, A. flavus S_B, A. flavus S_BG, A. bombycis, A. nomius, and A. pseudotamarii isolates, respectively, whereas the A. parasiticus aflR coding region was 99, 99, 93, 87, 87, and 89% identical to the coding regions of these isolates.

### 3.3. aflR Promoter structure

Alignment of the aflJ/aflR intergenic region (the sequence between the divergently transcribed aflJ and aflR ORFs) from the 28 isolates revealed putative binding sites for homologs of the known fungal transcription factors, AreA, AbaA, BrlA, PacC, and AflR (Fig. 1, Tables 3 and 4). The intergenic regions of all of the isolates contained at least two HGATAR sites (Table 3). In A. parasiticus, A. flavus S_B and L, N16, and A. pseudotamarii isolates two of the HGATAR sites were separated by 15 bp. Such adjacent sites have previously been shown to be sufficient to activate transcription by AreA (Ravagnani et al., 1997). A study previously showed that A. parasiticus AreA bound to DNA fragments from the A. parasiticus aflJ/aflR intergenic region that contained either GATA sites 1 and 2, or the single site. BrlA site 1 is within 100 bp of the aflR ORF. The region 30- to 90-bp upstream of the A. parasiticus aflR transcription start site (at ~33 bp) was previously found to be required for aflR promoter activity in reporter assays (Ehrlich et al., 1999a), but no sequence homologous to proven AflR-binding sites was present in this region.

Sequences previously shown to be sufficient for binding BrlA, a transcription factor involved in developmental regulation in A. nidulans (Adams et al., 1988; Adams et al., 1990), were present in all isolates except A. flavus S_B, and L. Some A. nomius isolates (Clade II) have two putative BrlA sites, whereas the others have only one site. BrlA site 1 is within 100 bp of the aflJ ORF. Putative sites for another factor shown to be important for developmental regulation in fungi, AbaA (Andriopoulos and Timberlake, 1994), were located upstream of the BrlA sites. The furthest upstream ABA site (site 1) was conserved in all isolates, but the second ABA site (site 2) was absent in A. flavus L, S_B, S_BG, and A. parasiticus isolates.

### Table 3

Presence of putative binding sites for AreA in the aflJ/aflR intergenic region

<table>
<thead>
<tr>
<th>Isolate*</th>
<th>aflJ/aflR Length (bp)</th>
<th>GATA1b</th>
<th>GATA2</th>
<th>GATA3</th>
<th>GATA4</th>
<th>GATA5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. flavus S_B (F3–F5)</td>
<td>739</td>
<td>-529</td>
<td>-508</td>
<td>-</td>
<td>-</td>
<td>-317</td>
</tr>
<tr>
<td>A. flavus L (F6–F9)</td>
<td>739</td>
<td>-528</td>
<td>-507</td>
<td>-</td>
<td>-</td>
<td>-316</td>
</tr>
<tr>
<td>A. parasiticus (P24–P26)</td>
<td>740</td>
<td>-529</td>
<td>-508</td>
<td>-</td>
<td>-</td>
<td>-317</td>
</tr>
<tr>
<td>A. flavus S_BG (F10–F12)</td>
<td>745</td>
<td>-509</td>
<td>-441</td>
<td>-</td>
<td>-</td>
<td>-319</td>
</tr>
<tr>
<td>A. pseudotamarii (PT27,PT28)</td>
<td>689</td>
<td>-493</td>
<td>-338</td>
<td>-</td>
<td>-</td>
<td>-322</td>
</tr>
<tr>
<td>A. bombycis (B1,B2)</td>
<td>719</td>
<td>-512</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-312</td>
</tr>
<tr>
<td>A. nomius (N16)</td>
<td>719</td>
<td>-509</td>
<td>-489</td>
<td>-</td>
<td>-</td>
<td>-310</td>
</tr>
<tr>
<td>A. nomius Clade I (N13,N14)</td>
<td>714</td>
<td>-</td>
<td>-487</td>
<td>-</td>
<td>-</td>
<td>-310</td>
</tr>
<tr>
<td>A. nomius Clade II (N15,N17–19, N23)</td>
<td>713</td>
<td>-</td>
<td>-485</td>
<td>-</td>
<td>-</td>
<td>-308</td>
</tr>
<tr>
<td>A. nomius Clade III (N20–N23)</td>
<td>719</td>
<td>-</td>
<td>-489</td>
<td>-</td>
<td>-</td>
<td>-312</td>
</tr>
</tbody>
</table>

*Letters refer to the species and numbers to the isolates listed in Table 1.

bProven binding sites for AreA have the sequence HGATAR or YTATCD. Numbering is from the aflR ORF. A ‘-’ indicates the site does not have the HGATAR binding motif.
A sequence identical to binding sites for the transcription factor involved in pH-regulation of gene expression in fungi, PacC (Espeso and Arst Jr, 2000), was found near the aflR ORF (PacC site 2) in A. parasiticus, A. flavus L, and A. bombycis and A. pseudotamarii isolates, but not in the A. nomius, or the A. flavus S_B and S_BG isolates. Another PacC site (site 1) was near the aflJ ORF. This site was present in most of the isolates, except for A. parasiticus, A. pseudotamarii, and the A. nomius isolates from Benin (N20–22).

3.4. aflR Coding region structure

Comparison of the deduced AflR protein sequence showed the presence of highly conserved domains homologous to nuclear localization signal sequences (RRARK, aa20–24) (Boulikas, 1993), the Cys6Zn2 motif (aa29–56), and a linker region necessary for sequence-specific DNA-binding (aa57–66) (Reece and Ptashne, 1993). Several of the most variable features in the protein sequence that might influence protein activity and/or abundance are listed in Table 5. The AflR protein sequences of A. flavus SB, A. pseudotamarii, and A. parasiticus isolates contained longer histidine-rich motifs (HAHTQAHTHAHSH, aa103–113) than did the other isolates. This motif was on the carboxy-terminal side of the DNA-recognition domain (Fig. 1). AflRs from A. flavus SBG isolates had only two of the six histidine residues, whereas the AflRs from A. bombycis and A. nomius had four of the His residues. In all isolates, a proline-rich region was adjacent to this site on the C-terminal side. AflR in A. nomius isolates had 11–12 proline residues while A. flavus and A. parasiticus isolates had eight.

A key distinguishing feature shared by A. nomius and A. bombycis isolates is a serine-rich sequence, NSSDSSGSSSSSSSSSSNNSP, approximately 100 amino acids from the AflR C-terminus and immediately preceding a conserved domain rich in acidic amino acids. Seven serine residues were present in the homologous region from A. pseudotamarii and four in A. flavus and A. parasiticus. Using the PESTfind algorithm (Rogers et al., 1986) the proline-rich sequence in A. nomius isolates had a score of +15 while the serine-rich sequence in A. nomius and A. bombycis isolates had scores of +18 and +12, respectively. In all isolates the final 20 amino acids at the AflR C-terminus, which may be part of the transcription activation domain (Matsumiya et al., 2001), is divided into an Arg-rich region (RH/QLRA/VVSS) and a region (DN/NI/D/V/LQ/HQ/QE) containing both basic and acidic amino acids.

3.5. Phylogenetic analysis

The trees based on the aligned nucleotide data sets for the aflJ/aflR intergenic region and coding region
sequence (Fig. 2) showed similarities in clades of taxa at distal nodes. In the tree based on intergenic region sequence, *A. nomius* isolates only separated into two distinct clades within which many of the affiliations were found to be polytomous (Fig. 2A) whereas in the tree obtained with coding region sequence (Fig. 2B), *A. nomius* isolates (excluding N16) were resolved into three well-supported clades.

When partitions consisting of the *aflJ/*aflR intergenic region and the *aflR* coding region sequence data sets were examined using the PHT, these regions were only significantly incongruent 
\(P < 0.05\) when specific *A. nomius* and *A. bombycis* isolates were included in the analysis (Table 6). The KH test (Kishino and Hasegawa, 1989) was used to compare the likelihood of the intergenic region sequence data given the topologies of the trees based on this data with the likelihood of the intergenic region data given the topologies of the trees based on the coding region sequence. The eight most parsimonious (MP) trees for the intergenic region were significantly less likely topologies for the coding region data set than any of the MP trees for the coding region (\(P < 0.001, -\ln L = 4502.72, \text{ and } \text{diff} - \ln L = 154\)). Similarly, the six MP trees for the coding region had less likely topologies for the intergenic region data set than did the MP trees for the intergenic region (\(P < 0.05; -\ln L = 2771.29, \text{ and } \text{diff} - \ln L = 32\)).

Using a simplified data set consisting of only one member of each taxon (B1, F3, F6, F10, P24, N15, and PT27), an attempt was made to determine if a phylogeny based on transcription factor binding sites maps to a phylogeny based on all parsimony informative *aflJ/*aflR intergenic region sites. All of the variable transcription factor binding site domains probably resulted from single nucleotide changes within the site. By PHT, the trees based on the transcription factor binding sites were

### Table 5

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Coding region motif&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ser-rich site 329</th>
<th>C-terminal sequence 427</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. flavus</em>, S9 and I (F3–F9)</td>
<td>hah, tgh, hsh, shh, pqp, npt, nsp, rhr, nft</td>
<td>nsg, ssnsp, rhr, vshlylre</td>
<td>rhr, vshlylre</td>
</tr>
<tr>
<td><em>A. parasiticus</em> (P24–P26)</td>
<td>hah, tgh, hsh, shh, pqp, npt, nsp, rhr, nft</td>
<td>nsg, ssnsp, rhr, vshlylre</td>
<td>rhr, vshlylre</td>
</tr>
<tr>
<td><em>A. flavus</em>, S9 (F10–F12)</td>
<td>hah, tgh, hsh, shh, pqp, npt, nsp, rhr, nft</td>
<td>nsg, ssnsp, rhr, vshlylre</td>
<td>rhr, vshlylre</td>
</tr>
<tr>
<td><em>A. pseudotamarii</em> (PT27,PT28)</td>
<td>hah, tgh, hsh, shh, pqp, npt, nsp, rhr, nft</td>
<td>nsg, ssnsp, rhr, vshlylre</td>
<td>rhr, vshlylre</td>
</tr>
<tr>
<td><em>A. bombycis</em> (B1,B2) and N16</td>
<td>hah, tgh, hsh, shh, pqp, npt, nsp, rhr, nft</td>
<td>nsg, ssnsp, rhr, vshlylre</td>
<td>rhr, vshlylre</td>
</tr>
<tr>
<td><em>A. nomius</em> Clade I (N13–14)</td>
<td>hah, tgh, hsh, shh, pqp, npt, nsp, rhr, nft</td>
<td>nsg, ssnsp, rhr, vshlylre</td>
<td>rhr, vshlylre</td>
</tr>
<tr>
<td><em>A. nomius</em> Clade II (N15,17–19,23)</td>
<td>hah, tgh, hsh, shh, pqp, npt, nsp, rhr, nft</td>
<td>nsg, ssnsp, rhr, vshlylre</td>
<td>rhr, vshlylre</td>
</tr>
<tr>
<td><em>A. nomius</em> Clade III (N20–22)</td>
<td>hah, tgh, hsh, shh, pqp, npt, nsp, rhr, nft</td>
<td>nsg, ssnsp, rhr, vshlylre</td>
<td>rhr, vshlylre</td>
</tr>
</tbody>
</table>

<sup>a</sup> Consensus sequence is shown. Sites are shown schematically in Fig. 1.
<sup>b</sup> Positions are the approximate number of amino acids from the protein’s amino terminus.
congruent with trees based on the entire intergenic region ($P = 1.000$). However, the KH test indicated that the topology of the MP tree for the transcription factor sites was not as good a description of the entire intergenic region data set ($P < 0.001$ and $\text{diff} / \text{ln} L = 93$).

### 4. Discussion

Although no study has demonstrated that aflatoxin production confers a selective advantage on producing organisms (Cotty et al., 1994), diverse Aspergillus species retain the ability to produce either aflatoxin or its precursors, a process that requires conservation of a gene cluster containing at least 23 different genes (Yu et al., 1995). Considerable differences in aflatoxin production among Aspergillus lineages have been found (Cotty and Bhatnagar, 1994). Both A. parasiticus and A. flavus Sb belong to clades in which aflatoxin-producing ability is highly conserved whereas A. nomius and A. flavus L isolates belong to clades in which aflatoxin production is highly variable (Cotty, 1989; Geiser et al., 2000). The radiation of ancestral Aspergillus species required adaptation to diverse animal and plant-associated niches in geographically isolated environments (Bayman and Cotty, 1993; Cotty and Bhatnagar, 1994). Considerable variability in numerous traits, including aflatoxin production, probably developed during this radiation. This report describes how the structure of aflR, the pathway-specific regulatory gene for aflatoxin biosynthesis, has evolved during divergence of aflatoxin-producing taxa within Aspergillus section Flavi, as inferred from 28 representative isolates. Sequence differences in the aflR coding and promoter regions provide a basis for predicting the roles of environmental and developmental cues in differential regulation of aflatoxin production among aflatoxin-producing fungi.

#### 4.1. Promoter sequence diversity

Adaptation to different environmental niches is evidenced by the diversity in binding sites for transcription factors sensitive to environmental stimuli in the promoter regions of the two regulatory genes involved in aflatoxin production, aflR and aflJ. Inhibition or stimulation of aflatoxin production by different nitrogen sources may vary with species, depending on the presence or absence of AreA-binding sites in their aflJ/aflR intergenic regions (Ehrlich and Cotty, 2000). Nitrate increases the availability of AreA, which affects the expression of genes depending on the number and position of promoter GATA sites (Muro-Pasteur et al., 1999). The variability of GATA sites in the aflJ/aflR intergenic region (Table 3) and differences among fungi in aflatoxin production (Table 1) provides a way to test the role of nitrogen sources on transcriptional regulation of aflatoxin biosynthesis genes.

**Table 6**

<table>
<thead>
<tr>
<th>Taxa included in analysis</th>
<th>Number of taxa</th>
<th>$P^b$</th>
<th>MP tree length$^c$</th>
<th>Min. tree length$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A All taxa</td>
<td>28</td>
<td>0.026</td>
<td>730</td>
<td>723</td>
</tr>
<tr>
<td>B All taxa excluding A. nomius</td>
<td>19</td>
<td>0.291</td>
<td>648</td>
<td>646</td>
</tr>
<tr>
<td>C All taxa excluding N20–22</td>
<td>25</td>
<td>0.492</td>
<td>697</td>
<td>694</td>
</tr>
<tr>
<td>D All taxa excluding N13, N14</td>
<td>26</td>
<td>0.682</td>
<td>702</td>
<td>699</td>
</tr>
<tr>
<td>E All taxa excluding N15,17–19, 23</td>
<td>23</td>
<td>0.054</td>
<td>677</td>
<td>674</td>
</tr>
<tr>
<td>F All taxa excluding N16, B1, B2</td>
<td>25</td>
<td>0.097</td>
<td>602</td>
<td>594</td>
</tr>
<tr>
<td>G A. nomius (excluding N16)</td>
<td>10</td>
<td>0.280</td>
<td>104</td>
<td>103</td>
</tr>
<tr>
<td>H G + A. bombycis and N16</td>
<td>13</td>
<td>0.012</td>
<td>312</td>
<td>307</td>
</tr>
<tr>
<td>I A. flavus L and Sb</td>
<td>7</td>
<td>1.000</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>J I + A. parasiticus</td>
<td>10</td>
<td>0.301</td>
<td>66</td>
<td>63</td>
</tr>
<tr>
<td>K J + A. flavus Sb + A. pseudotamarii</td>
<td>15</td>
<td>0.630</td>
<td>335</td>
<td>331</td>
</tr>
</tbody>
</table>

$^a$Partitions are aflJ/aflR intergenic region and aflR coding region.  
$^b$Probability is based on 1000 bootstrap replicates.  
$^c$MP, most parsimonious.  
$^d$Tree lengths include phylogenetically informative sites only. Min., minimum.
processes as sporulation or sclerotial morphogenesis (Cotty, 1988; Hicks et al., 1997). High aflatoxin production has been associated with phenotypes having increased sclerotial formation, particularly in the A. flavus S5 morphotype (Cotty, 1997; Geiser et al., 2000).

Aflatoxin biosynthesis also is markedly influenced by pH (Cotty, 1988; Keller et al., 1997). Presence or absence of PacC-binding sites in the aflJ/aflR intergenic region could partly account for differential sensitivity of aflatoxin production to pH in different species of Aspergillus (Ehrlich et al., 1999a). Precedent for interference by PacC in the expression of acid-expressed genes has been reported for the gabA gene in A. nidulans (Espeso and Arst Jr, 2000). The proximity of PacC site 1 to the aflJ ORF and PacC site 2 to the aflR ORF suggests that they may be involved in pH regulation of expression of these genes. Variation among Aspergillus isolates in retention of these PacC sites provides an opportunity for empirical evaluation of the role of PacC in transcriptional control of aflR.

A consensus AflR-binding site near the aflJ ORF (Table 5) is present in all isolates except A. nomius, suggesting that, aflJ, like most of the genes in the aflatoxin biosynthetic pathway, is usually regulated by AflR. A. bombycis and A. pseudotamarii isolates have a second AflR recognition site slightly further upstream. Since aflJ is also involved in regulation of aflatoxin pathway biosynthesis (Chang et al., 2000; Ehrlich et al., 1999b; Meyers et al., 1998), variability in regulation of aflJ could serve as an important checkpoint on production of aflatoxins.

4.2. Coding region diversity

As expected, several portions of the coding region were highly conserved among all of the isolates examined. These included the DNA-binding domain [Cys4Zn2 sequence and linker region (Ehrlich et al., 1998)] and domain(s) within 60 amino acids of the carboxy terminus shown to be required for transcription activation (Chang et al., 1999; Matsushima et al., 2001). The His-rich region that occurs after the linker region varies in size among the different species. Such histidine repeat regions are found in a number of eukaryotic transcription factors and may be involved in pH-controlled protein–protein interactions (Janknect et al., 1991). Differences in length of these repeats among AflRs could be important in determining AflR activity at different pHs. The serine-rich region (Fig. 1, Table 5) in AflRs from A. nomius and A. bombycis is a distinct PEST (proline, glutamine, serine, and threonine-rich region) sequence that may be a target for phosphorylation and ubiquitin-mediated proteolysis (Rechsteiner, 1988). A. nomius isolates (except N16) have a second PEST domain (HPPPPPPQSDQPQPH, PEST score = +15). No comparable region exists in AflRs from other species. PEST scores for these regions (+12 to +18) were comparable to scores of +10 to +13 for known PEST domains in other transcription factors (Suske, 1999). Most proteins with PEST sequences are regulatory molecules that require fast turnover for proper function. The PEST sequences in A. nomius and A. bombycis either may reflect regulatory mechanisms different from other aflatoxin-producing taxa or may be non-functional remnants of an ancestral mechanism to regulate cellular levels of AflR at the post-transcriptional level. Ancesters of A. flavus and A. parasiticus may have developed strategies to control protein production at the transcriptional level by developing in their promoter regions increased numbers of certain transcriptional modulatory sites sensitive to the environment, such as GATA and PacC sites. The latter strategy avoids the production of excess protein and may provide an energy advantage over fungi lacking such sites.

4.3. Phylogenetic analysis

In general, phylogenetic analyses inferred clades corresponding to previously described species and/or strains (Cotty and Cardwell, 1999; Ito et al., 2001; Peterson et al., 2001). Similar distal monophyletic groupings were obtained with either the aflJ/aflR intergenic region sequence or inferred AflR protein sequence data sets. Comprehensive surveys of phylogenetic relationships in Aspergillus section Flavi (using 5S RNA internal transcribed spacer, ITS, sequence data and a distantly related species as the outgroup) suggested that A. nomius diverged prior to A. parasiticus, A. pseudotamarii, and A. flavus (Peterson, 1997; Ito et al., 2001; Peterson et al., 2001). A comparison of A. bombycis and A. nomius in which the non-aflatoxin producing species A. carbonarius was chosen as the outgroup, suggested that A. bombycis diverged prior to A. nomius (Peterson et al., 2001). Based on these comparisons we chose A. bombycis as the outgroup in the present study. If A. parasiticus was selected as the outgroup, the trees obtained showed phylogenetic relationships for A. flavus isolates closely resembling those previously found (Geiser et al., 1998, 2000).

Tree topologies for the aflJ/aflR intergenic and the aflR coding regions were significantly incongruent based on the PHT, but only when certain A. nomius clades and A. bombycis were included in the analysis. Incongruence was not found when A. nomius isolates were examined independently from the other taxa (Table 6). In a study of incongruence and phylogenetic accuracy, Cunningham found that when the PHT resulted in $P > 0.01$, the combined data better reflected the phylogenetic signal than the data of the individual partitions (Cunningham, 1997). Thus, from the present PHT results (Table 6), trees based on the combined coding region and intergenic region data sets are probably the best representation of
the phylogenetic signal within the examined data. The apparent incongruence observed here between the coding region and intergenic region data sets with PHT was supported by the results of the KH test. The KH test indicated that the topology of the intergenic region tree was not a good fit for the coding region data set. These tests suggest that there may be differences between the phylogenetic signals within the two data sets. The differences may have resulted from differential convergence or transfer of genetic material between closely related clades of *A. nomius* and possibly, between *A. nomius* and *A. bombycis*. Peterson had previously made similar observations within *A. nomius* (Peterson et al., 2001). The need to express genes for aflatoxin biosynthesis in new environments may require adaptation via changes in promoter sequence that allow recognition by transcription factors that mediate response to environmental signals. Indeed, the topology of the MP tree for transcription factor recognition sites is not a good fit for the entire intergenic region data set by the KH test. Convergence among promoter elements may have occurred while the non-regulatory portions of the intergenic region retained divergent characters.

Phylogenetic affiliations not described by current taxonomic schemes were also discovered in our analyses. An example is isolate N16 from Brazil nut imported into the US. This isolate was previously reported as a divergent *A. nomius* (Egel et al., 1994). Sequence comparison indicates that N16 is more closely related to *A. bombycis*. However, it is not clear that isolate N16 fits entirely within the *A. bombycis* species concept. Although N16 has smooth stipes and spores similar to *A. bombycis*, it produces large, elongate sclerotia (illustrated in Cotty et al., 1994)), whereas *A. bombycis* produces no sclerotia (Peterson et al., 2001). N16 would be the only *A. bombycis* isolate from a plant and the only one originating from South America. Isolate N16 shares highly supported clades with the *A. bombycis* isolates in both the intergenic region DNA and protein sequence trees, but differs in some regulatory features such as having seven GATA sites versus the five in *A. bombycis* isolates. Isolate N16 may belong to a South American lineage that has a relatively recent ancestor in common with *A. bombycis*. Differences in potentially adaptive traits (sclerotial morphology, aflatoxin regulation) may indicate that the lineage of N16 was exposed over time to different selective pressures than the lineage leading to the previously described *A. bombycis* isolates, as might be expected for an isolate from plant compared to isolates from insect debris.

*A. nomius* isolates, other than N16, formed several distinct clades in both trees. In agreement with previous observations (Feibelman et al., 1998), the three *A. nomius* O-strain isolates (N17–19), which are morphologically and physiologically distinct from other *A. nomius* strains, were placed in the same clade as the expected type *A. nomius* (N15). However, the O-strain isolates accumulate less aflatoxin than the other isolates in this clade. Some of the North American isolates are more closely related to isolates from either the Philippines (N23) or West Africa (N20–22) than to two isolates from Mississippi (N13 and N14). Although, delineation of a clade solely occupied by the three *A. nomius* from West Africa (N20–22) reveals geographic structure and possible allopatric speciation, not all of the cladal separations of *A. nomius* are clearly allopatric.

Aflatoxin-producing species are commonly divided into those producing both B and G aflatoxins and those producing only B aflatoxins (Table 1). Our phylogenetic analysis suggests that the ability to produce both B and G aflatoxins is the ancestral trait and that loss of G aflatoxin production occurred at two separate times among extant *Aspergillus* lineages. Loss occurred once in the lineage leading to *A. pseudotamarii* and a second time in the lineage leading to *A. flavus* L and S. From the current data, we cannot determine if failure to produce G aflatoxins is through similar mechanisms for the two lineages.

The phylogenetic analyses presented here separate *A. flavus* S and L isolates into distinct clades in agreement with previous analyses (Bayman and Cotty, 1991; Cotty, 1997; Geiser et al., 2000), and also reinforce previous observations that these *A. flavus* morphotypes are far less closely related to the S, G isolates than to each other or to *A. parasiticus* (Egel et al., 1994). RAPD polymorphisms, sclerotial morphology, and the differential ability to produce aflatoxins (Bayman and Cotty, 1991; Cotty, 1997) have also supported delineation of S and L isolates into separate clades. Results of the current study concur with previous results which suggested that the S, G isolates are distinct from *A. flavus* (Hesseltine et al., 1970; Egel et al., 1994; Geiser et al., 2000). Efforts should be made to place S, G isolates into a systematically distinct taxon.

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References

Aspergillus nidulans


remodelling in a eukaryotic bidirectional promoter. EMBO J. 18, 1584–1597.


Rambo, G.W., Tuite, J., Crane, P., 1974. Preharvest Inoculation and infection of Dent corn ears with Aspergillus flavus and A. parasiticus. Phytopathology 64, 797–800.


