

## PERMANENT GENETIC RESOURCES NOTE

# Twenty-four microsatellite markers for the aflatoxin-producing fungus *Aspergillus flavus*

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**Abstract**

*Aspergillus flavus* infects both plants and humans and contaminates diverse agricultural crops with aflatoxins, highly carcinogenic fungal metabolites. We describe 24 microsatellite markers developed to assess genetic diversity and recombination within and between three vegetative compatibility groups (VCGs) of *Aspergillus flavus*. These loci are polymorphic within at least one VCG or between VCGs. For loci polymorphic across all three VCGs, the number of alleles ranged from two to 19. These markers will be useful for genetic studies of this economically important pathogen.

**Keywords:** Ascomycota, asexual, clonal, haploid, mycotoxin

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The haploid fungus *Aspergillus flavus* is a widely distributed opportunistic pathogen of both animals and plants; however, *A. flavus* is best known as the primary cause of aflatoxin contamination. Aflatoxins are fungal metabolites that damage DNA, cause cancer at minute concentrations, and impair child development. *A. flavus* has a vegetative incompatibility system that limits gene flow among dissimilar individuals but no known sexual stage. *A. flavus* reproduces and disperses clonally via mitospores and populations have a high diversity of vegetative compatibility groups (VCG). Population genetics of *A. flavus* is largely undescribed as a result of the difficulty of developing molecular markers that are polymorphic among and within the very closely related VCGs. Our objective is to develop molecular markers suitable for investigating levels of genetic diversity and recombination within and between *A. flavus* VCGs.

Microsatellite loci with di- to hexanucleotide repeats and 1000-bp flanking sequence were identified from the genome sequence of *A. flavus* NRRL3357 (<http://www.aspergillus-flavus.org/>) using Tandem Repeats Finder version 4.00 (Benson 1999). Primers were designed for 68 loci with amplicons > 700 bp using Primer 3 (Rozen & Skaletsky 2000). Polymorphism was assessed by sequencing four isolates from each of three *A. flavus* VCGs (CG136, MR17,

OD02). Genomic DNA was extracted from mycelial cultures using the FastDNA SPIN kit (QBI0-gene). Twenty microlitres polymerase chain reactions (PCR) utilized Bioneer AccuPower Hotstart PCR PreMix with 1× PCR buffer with 1.5 mM MgCl<sub>2</sub>, 1.0 U HotStart *Taq* DNA polymerase, and 250 μM of each dNTP, 3–10 ng of DNA, and 0.3 μM of each primer. Products were amplified with thermocycler parameters: 95 °C for 15 min; 10 cycles of 94 °C for 30 s, 62 °C decreasing 1 °C per cycle for 30 s, 72 °C for 60 s; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s; and 10 min at 72 °C. PCR amplicons were sequenced bidirectionally on an ABI 3730xl DNA Analyser (Applied Biosystems) at the University of Arizona Genomic Analysis and Technology Core Facility (GATC). Contigs were assembled automatically using Phred and Phrap (Green 1999; Green & Ewing 2002) and edited using ChromaSeq (Maddison & Maddison 2005) in Mesquite (Maddison & Maddison 2006).

Primers for 24 loci were designed for shorter PCR amplicons for use in genotyping isolates (Table 1). Polymorphism was estimated from 221 isolates from Arizona and Texas of VCGs CG136, MR17 and OD02 (Table 2). One primer was 5'-fluorescently labelled for use with the ABI G5 dye set. Multiplex PCR was carried out using QIAGEN Multiplex PCR Kit in a 10 μL final volume with 1× QIAGEN Multiplex PCR Master Mix, 0.25× Q-solution, 0.1 μM of each primer and 1–5 ng DNA. Thermocycler conditions were: 95 °C for 15 min, 25 cycles of 94 °C for 30 s, 57 °C for 90 s, 72 °C for 30 s, and 30 min at 60 °C. PCR amplicons were sized on

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Locus name	Primer sequence (5'-3')	Repeat motif and scaffold	Size (bp)
AF8	F: <i>PET</i> -GGCTTGCAAGTCTAATCTGC R: TGTGTCTTTGGGATGTATTTTCG	(AAG) <sub>16</sub> 2911	178
AF10	F: <i>6FAM</i> -CGTGCCATCGTAGAACTTCC R: GGGACATTGGTAGTACCTTGG	(TAC) <sub>10</sub> 2504	274
AF11	F: <i>NED</i> -GACGGCGGTGTACAGTGATAGT R: GCAGTAACGCGATTATGCAAGT	(AAG) <sub>12</sub> 2504	142
AF13	F: <i>VIC</i> -CGTGTTCCAAGTCAAGTCCA R: TCTCCTTTGCTCCCGTTAGA	(CTT) <sub>9</sub> 1866	136
AF16	F: <i>6FAM</i> -AGGTCGTGAAGCCGATACTG R: CAAAGGCAGATCGAAGGGTA	(TTG) <sub>10</sub> 2541	179
AF17	F: <i>VIC</i> -CTATAGAGGTGGCGCAGAGG R: GGTTTCTCGTCTCGTCTTTG	(AGA) <sub>4</sub> (AGG) <sub>10</sub> 1918	366
AF18	F: <i>VIC</i> -ACTGAGCATTACCTGCTTG R: ACCTAGCGGGAGGTTCTAGG	(TTC) <sub>29</sub> 1918	198
AF22	F: <i>NED</i> -TCACATGCTTGACCTTGGAT R: TTGCCAACTCATCACTCACG	(TTTA) <sub>8</sub> 2911	185
AF25	F: <i>NED</i> -CCGCTCCGAGTGTACTTA R: CAATAAGGATCGCAATCGTACA	(TAC) <sub>7</sub> TCC(TAC) <sub>4</sub> 2504	304
AF25_MR17	F: <i>NED</i> -GCTAGAAGGATACGCGTGGT R: CGAATCAGGCATTGGCTACT	Same as AF25	325
AF26	F: <i>PET</i> -GGCGGGTGTAAAGATGAGAA R: GGGATGAAATGAATGCCTCA	(AAT) <sub>15</sub> 2504	377
AF27	F: <i>PET</i> -TTGCCGTCTTTATCGATTAGC R: CTTTCAAGGGATGGGAGTGA	(GTT) <sub>14</sub> 2504	288
AF28	F: <i>6FAM</i> -CCTCCCAATTCACCACTCACT R: TTCCTGGGAAGTGAAGAACG	(TTG) <sub>11</sub> 2504	161
AF31	F: <i>PET</i> -GGGTTGCTTTGAGGTGTGAG R: GGAGTGGCTAGATCGCATGT	(TTC) <sub>31</sub> 2634	377
AF33	F: <i>VIC</i> -ATTGTTGCCTTCTCCGTCCAC R: CAGGCGATATTTGCATCAGA	(AGT) <sub>3</sub> AGC(AGT) <sub>13</sub> 2911	176
AF34	F: <i>VIC</i> -CAGTCAACCTTGGCATCGTA R: ACCAAACCCAAACCCTAACC	(GTC) <sub>4</sub> (GTT) <sub>8</sub> 2911	306
AF42	F: <i>6FAM</i> -TCTGATGCGCAAGCTGTAAG R: TCTGCCAAGCAAGAGGAAGT	(TTC) <sub>16</sub> 2634	168
AF43	F: <i>VIC</i> -GTGAGAGCAATTGGGAAACC R: TGACCAATATGCTGGAGGTG	(GAG) <sub>13</sub> 2634	392
AF48	F: <i>VIC</i> -CCACGTTCCACTGTCTCCCT R: GCAAGTCTCCACTGATGGT	(AAG) <sub>12</sub> 2802	352
AF53	F: <i>6FAM</i> -TCCTCCAAAGTGACCAAAGC R: TGGCATTGCTCAGGACATAG	(TCT) <sub>8</sub> 1918	147
AF54	F: <i>VIC</i> -GAGAGGTATGCCTTCATGCTTT R: AGTGTGTCGACATGGATTGC	(ACAT) <sub>8</sub> 1918	173
AF55	F: <i>PET</i> -TCATGATCAACCCAGTCCAA R: TGGGCAGAAATATCCACGTCT	(GT) <sub>10</sub> 1739	169
AF63	F: <i>NED</i> -CTGGTCGCTGGAAGTATTT R: GATGTTGGCTGCCGTTAGTT	(AT) <sub>7</sub> 2856	134
AF64	F: <i>VIC</i> -GCCTAAGGACGAGTCGATTG R: GAGGACCGAAGAAGGATGTG	(AC) <sub>16</sub> 2856	177
AF66	F: <i>6FAM</i> -TCCCGTGTGTCACCTGTTGTT R: GAGGTAGCCATGGAAACTGG	(AT) <sub>12</sub> 1569	265

**Table 1** Characterization of 24 microsatellite loci developed for *Aspergillus flavus* with primer sequences, repeat motif, scaffold, and size (number of base pairs, bp) in the genome sequence *A. flavus* NRRL3357

an ABI 3730 DNA Analyser at GATC with the LIZ500 standard (Applied Biosystems). Allele sizes were called using GeneMarker version 1.6 (SoftGenetics LLC). Reproducibility of genotyping results was confirmed by running

three to five independent PCR and genotyping runs on at least 10% of the isolates in each VCG. To confirm whether length polymorphism was due to microsatellite repeat number representative alleles were sequenced using the

**Table 2** Haploid diversity for the 24 microsatellite loci developed for *Aspergillus flavus* vegetative compatibility groups (VCG) CG136, MR17 and OD02. For each VCG, number of isolates genotyped from Arizona and Texas populations combined ( $n$ ), number of alleles ( $N_A$ ), range of allele sizes in number of base pairs (bp), and haploid gene diversity ( $h$ ) are shown. Loci are polymorphic within at least one VCG or between VCGs

Locus	VCG CG136				VCG MR17				VCG OD02			
	$n$	$N_A$	Size (bp)	$h$	$n$	$N_A$	Size (bp)	$h$	$n$	$N_A$	Size (bp)	$h$
AF8	75	5	160–188	0.22	84	10	157–212	0.62	62	3	173–179	0.12
AF10	75	8	282–305	0.64	84	19	293–413	0.88	62	10	279–348	0.84
AF11	75	16	132–233	0.86	84	11	156–230	0.71	62	4	123–159	0.13
AF13	75	7	137–166	0.25	84	1	125	0.00	62	3	122–160	0.47
AF16	75	3	187–193	0.20	84	1	175	0.00	62	2	181–184	0.09
AF17	75	2	353–356	0.03	84	5	358–397	0.22	62	3	367–373	0.06
AF18	75	3	154–160	0.08	84	2	151–154	0.05	62	0	—	—
AF22	75	1	179	0.00	84	2	183–191	0.02	62	1	199	0.00
AF25*	75	2	305–308	0.08	84	1	329	0.00	62	2	308–311	0.09
AF26	75	1	348	0.00	84	6	404–434	0.59	62	1	348	0.00
AF27	75	1	262	0.00	84	1	262	0.00	62	2	280–283	0.27
AF28	75	4	121–144	0.15	84	2	135–138	0.07	62	2	115–118	0.03
AF31	75	2	321–324	0.05	84	13	327–414	0.81	62	2	308–311	0.03
AF33	75	2	174–177	0.03	84	1	174	0.00	62	2	171–174	0.06
AF34	75	2	316–319	0.05	84	1	300	0.00	62	5	329–342	0.49
AF42	75	6	162–214	0.29	84	11	159–240	0.74	62	5	162–177	0.48
AF43	75	2	384–387	0.08	84	3	384–390	0.07	62	4	384–393	0.41
AF48	75	4	350–364	0.10	84	4	367–378	0.09	62	11	367–400	0.75
AF53	75	3	150–172	0.08	84	2	144–147	0.48	62	1	134	0.00
AF54	75	2	161–165	0.03	84	2	173–177	0.02	62	2	177–181	0.03
AF55	75	2	180–182	0.13	83	2	190–192	0.16	62	1	172	0.00
AF63	75	1	127	0.00	83	1	131	0.00	62	1	135	0.00
AF64	75	6	187–203	0.58	83	2	171–173	0.09	62	14	169–221	0.88
AF66	75	1	269	0.00	83	1	269	0.00	62	2	269–271	0.18

$n$ , clone-corrected sample size.

$h = (n/n - 1) \times (1 - p_i^2)$ , where  $p_i$  is the frequency of the  $i$ th allele for the population and  $\sum p_i^2$  is the sum of the squared population allele frequencies.

\*AF25\_MR17 primers (Table 1) were used for VCG MR17.

initial primers designed for all VCGs and all loci (GenBank Accessions EU877537–EU877626).

Haploid diversity was calculated for each VCG separately (Table 2) using GenAlix Version 6.1 (Peakall & Smouse 2006). All 24 loci were polymorphic within at least one of the VCGs screened or between VCGs. The number of polymorphic loci varied from 16 in VCG MR17 to 19 in CG136. The number of alleles per locus varied from two to 19 across VCGs. Null alleles were not detected, except that AF18 did not amplify in isolates of OD02 with both sets of primers. In all cases, reproducibility tests confirmed genotype data. Haploid gene diversity ranged from 0.00 to 0.88 per locus across VCGs. Allele sizes at five loci are not comparable across VCGs because length variation is affected by a mutation that changed the repeat motif in one repeat at the beginning or end of the microsatellite (AF10, AF26, AF27, AF48), or for AF25, primers for MR17 were designed due to nucleotide polymorphisms in the flanking region.

These five loci are suitable for use within at least one VCG, or can be used between VCGs using repeat number.

Linkage disequilibrium between pairs of polymorphic loci was calculated separately for each VCG using Multi-locus version 1.3b (Agapow & Burt 2001). Significance was assessed with 999 permutations and was not significant after Bonferroni correction for multiple comparisons ( $\alpha' = 0.05/\text{number of polymorphic loci for each VCG}$ , Table 2) with the exception of two loci. Evidence of significant linkage disequilibrium was observed in MR17 locus AF17 with 10 loci (AF18 through AF42, Table 1); and in OD02 locus AF10 with 16 loci (AF11 through AF48, Table 1). Significant linkage disequilibrium is not likely a result of proximity on the same chromosome because most loci were located on different scaffolds (Table 1). Possible explanations include association due to clonal reproduction and undetected fine-scale geographical structure and these will be addressed through further study.

The microsatellite markers reported here are the first developed for *A. flavus* VCGs and they will be useful in assessing recombination and genetic diversity in a fungus with significant economic and human health importance.

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