Molecular Ecology Notes (2004) **4**, 215–218 doi: 10.1111/j.1471-8286.2004.00621.x

PRIMER NOTE

Development and characterization of microsatellite markers for the fungus *Ceratocystis fimbriata*

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Abstract

***Ceratocystis fimbriata* is a serious fungal pathogen on a wide range of plants, but many cryptic species within *C. fimbriata* are apparently host-specialized. Anchor polymerase chain reaction (PCR) and simple sequence repeat (SSR) enriched libraries were used to develop 16 microsatellite markers for *C. fimbriata*. All markers were polymorphic when tested against isolates from four host-specialized lineages of the pathogen. These markers will be valuable for phylogenetic and population genetic studies, as well as for tracking accidental introductions of host-specialized forms of the pathogen.**

Keywords: Anchor PCR, *Ceratocystis*, *Ceratocystis fimbriata*, fungi, microsatellites, SSR

Received 9 Deceember 2003; revision received 16 January 2004; accepted 16 January 2004

Ceratocystis fimbriata is a fungal plant pathogen that attacks an exceptionally wide range of economically important hosts, including almond, cacao, coffee, eucalyptus, mango, sycamore, sweet potato, and taro (CAB International 2001). Several genetic lineages within the *C. fimbriata* complex are strongly host-specialized, and some of these lineages have caused serious epidemics after being moved beyond their native ranges by humans (Baker *et al*. 2003). Because of its economic importance and our studies on the genetic and ecological diversity within this species complex, it was desirable to develop genetic markers for analysing relationships within and among populations and for genotyping invasive strains. Santini & Capretti (2000) employed RAPDs and direct M13 primer amplification of minisatellite regions, and Barnes *et al*. (2001) developed PCR primers for polymorphic regions, some with simple repeat sequences. However, we desired highly polymorphic and reproducible markers, preferably with tri- or tetra-nucleotide repeats. Here, we depict the isolation and characterization of 16 microsatellite markers in four host-specialized lineages of the pathogen.

Isolates for DNA extraction were grown at 25 °C for 2 weeks in 20 mL of malt yeast extract broth (2% malt, 1% yeast). Total genomic, high molecular weight DNA was extracted from isolates as described by DeScenzo & Harrington (1994). Genomic DNA of isolate C1548 from *Theobroma cacao* in Costa Rica (Baker *et al*. 2003) was used for identification of microsatellite loci using modifications of either an anchor PCR technique (Fisher *et al*. 1996) or the enrichment technique of Edwards *et al*. (1996). Target motifs were chosen based upon previous experience (DeScenzo & Harrington 1994) or on genome survey data (Toth *et al*. 2000; Wostemeyer & Kreibich 2002).

For anchor PCR, four 27 bp primers of tri- or tetra-nucleotide repeat sequences with degenerative 5′-anchors were designed: DBV$(CAT)_8$, D.B.H.$(CAG)_8$, BBH$(AAG)_8$, and BDB$(GACA)_6$. The Universal Genomewalker kit (BD Biosciences, Palo Alto, CA) was utilized to further purify genomic DNA, to construct genewalking libraries, and for PCR amplification of microsatellite loci employing one of the anchor primers and the adapter primer AP1 from the kit. After a single round of PCR, products were separated by agarose electrophoresis, purified from the gel using a Geneclean II kit (BIO 101 Inc., Vista, CA), and cloned into the pGEM-T Easy Vector (Promega Inc., Madison, WI). Recombinant plasmids were extracted using the QIAprep Spin Miniprep plasmid DNA extraction kit (Qiagen Inc., Valencia, CA) and inserts screened for size by electrophoresis after restriction by *EcoR* I enzyme (Invitrogen, Carlsbad, CA). Clones with inserts of the expected size were sequenced at the Iowa State University DNA Sequencing and Synthesis facility using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA). For sequences with the proper simple sequence repeat, two reverse genewalking primers were designed on the 3′ flanking region using

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Table 1 Loci, primers, size of PCR product, and GenBank accession numbers for microsatellite markers developed from a cacao isolate of *Ceratocystis fimbriata* using the anchor PCR or Edwards techniques

Locus	Isolation technique	Sequence motif*	Primers	Label	Primer sequences	Actual size (bp) in isolate C1548	GenBank accession no.
CfAAG8	Anchor PCR	$(AAG)_{11}$	AAG8-1F AAG8-1R	TET	5′-TAG-ACA-GGG-GGT-GCG-TCA-AA 5′-TGT-CTG-CCC-TCC-ACA-TTT-GGT-CTC-TTC	187	AY494859
CfAAG9	Anchor PCR	$(CAG)_2$ + $(CAG)_7$ + $(AAG)_7$	AAG9-1F AAG9-1R	FAM	5′-CCT-GAA-CTG-ACA-GAG-ACA-CTT 5′-GCA-CCA-GCT-GTT-CCT-AAT-CGT	413	AY494860
CfCAA9	Edwards	$(CAA)_4(CAG)_2$ + $(CAG)_2$ + $(CAG)_6$ + $(CAG)_4$ + $(CAG)_2(CAA)_{20}$ $(CAG)_5$	CCAA9-F CCAA9-R	FAM	5′-GGC-TGG-TTC-ATC-ATG-ATG-TT 5′-CTA-TGG-CAC-CTA-AGC-AAT-CT	253	AY494861
CfCAA10	Edwards	$(CAA)_4(CAG)_6$	CCAA10-F CCAA10-R	HEX	5′-TGA-CAC-GCG-CTT-CAC-TAA-CAG 5′-TGC-ACC-ATA-CCC-AGG-GGA-CA	129	AY494862
CfCAA15	Edwards	$(CAA)_6(CAG)_8$ $(CAA)_2$ + $(CAA)_2$ + $(CAA)_2(CAG)_3$ $(CAA)_2$	CCAA15-F CCAA15-R	TET	5′-GCT-ACA-GCA-GCC-GCA-GTG 5′-GAT-TGG-CGT-TAG-TGT-TAG-GT	342	AY494863
CfCAA38	Edwards	$(CAG$ or $CAA)_{47}$	CAA38-1F CAA38-1R	FAM	5′-AAT-TCG-GGA-GCT-GCT-GTG-AG 5′-GAG-CCC-CAG-CCT-CAA-ACT-CA	240	AY494864
CfCAA80	Edwards	$(CAG$ or $CAA)_{43}$ + $(CAG)_3$ + $(CAG)_2$ + $(CAG)_2$ + $(CAA)_3$	CCAA80-F CCAA80-R	HEX	5′-ACC-CGT-CTC-GTA-TTG-GCT-AT 5′-AAT-CGT-TCG-CAT-TCA-GGT-GG	311	AY494865
CfCAT1	Anchor PCR	$(CAT)_{11}$ + $(CAT)_2$	DBVCAT1-1F WCAT1-1R	FAM	5′-CCC-AAT-TTC-CCA-TTC-TGA-TTC 5′-AGT-ACA-GGA-TCA-ACT-ATG-GCA-TTT-CAA	272	AY494866
CfCAT3K	Anchor PCR	$(CAT)_6$ + $(CAT)_2$	WCAT3K-1F WCAT3K-1R	FAM	5′-AGG-TGC-CCT-ACA-CTT-TTT-GAA 5′-GTT-TTG-CTT-CTG-GCT-ACT-TTG-GTA-CTG	330	AY494867
CfCAT9X	Anchor PCR	$(CAT)_6$	CAT9X-1F CAT9X-2R	HEX	5′-CCT-CGC-CTT-AAG-TTG-AAG-AAG 5′-GCG-GTT-GAG-GTT-GAA-GTG-TAG-AGT-GGT	273	AY494868
CfCAT1200	Anchor PCR	$(CAT)_7$	CAT12X-1F CAT12X-1R	TET	5′-ACA-AAA-GAC-GGC-ACG-CAT-ACA 5′-TGG-GGA-GAA-GTC-TGA-GTA-GAG-GGA-CAA	380	AY494869
CfCAG5	Anchor PCR	$(CAG$ or $CAA)_{12}$	CAGDL2-5-1F CAGDL2-5-1R	FAM	5′-AAG-CCC-GGT-TAC-AGA-AGC-AAG 5′-GTG-CTT-GAG-TTT-GTC-CAG-GGT-TCG-GTA	342	AY494870
CfCAG15	Edwards	$(CAG)_4(CAA)_2$ $(CAG)_4$ + $(CAG)_2$ + $(CAG)_3(CAA)_6$ $(CAG)_9$ + $(CAG)_2$ $(CAA)_{10}$ + $(CAG)_4$	CCAG15-F CCAG15-R	HEX	5′-GGG-CTA-GTA-GCA-GAG-TTG-G 5′-GCC-AAT-GTC-TTC-ACA-CCA-C	269	AY494871
CfCAG900	Anchor PCR	$(CAG)_4$ + $(CAG)_2$	CAG900-1F CAG900-2R	HEX	5′-CTT-TGC-TAG-TCC-CCA-GTT-CCA 5′-GCG-GAC-ATG-GGA-TTG-TAA-GAG-CCT-GAG	196	AY494873
CfGACA60	Anchor PCR	$(GACW)_4$ + $(CACAGCA)_4$	GACA60-1F GACA60-1R	TET	5′-GGC-GAC-GGC-AAA-TAG-CAA-AAT 5′-GAT-GTG-TGG-TGC-TGT-GGT-ATG-CTG-CTG	191	AY494874
CfGACA650	Anchor PCR	$(TG)_4$ + $(CA)_2$ $(GACA)_4$ + $(CCT)_2$	GACA6K-1F GACA6X-2R	HEX	5′-AAA-CAT-CTC-GGC-AGA-ACA-GC 5′-TGC-CGC-TTT-TGC-TTT-GTA-GTG-TTC-TTG	212	AY494875

*'+' indicates that two simple repeats were separated by other bases.

OLIGO version 5.1 (National Biosciences Inc, Plymouth MN), and genewalking was repeated per kit instructions to obtain the upstream flanking sequences. After the two flanking regions of the microsatellite repeat were sequenced, a new forward primer was designed and fluorescently labelled with either HEX, TET, or FAM (Integrated DNA Technologies, Iowa City, IA). This labelled primer was used with one of the reverse genewalking primers to amplify the microsatellite region.

Two microsatellite-enriched libraries (CAA and CAG) were also constructed using a modified protocol of Edwards *et al*. (1996). Oligonucleotides of either $(CAA)_{10}$ or $(CAG)_{10}$ were bound to nylon membranes, and the PCR library (Edwards *et al*. 1996) of isolate C1548 was hybridized to the membrane. After a series of washings of increasing stringency (Edwards *et al*. 1996), strongly hybridizing fragments were eluted, reamplified (using the adaptor primers of Edwards *et al*. 1996), and cloned into the pGEM-T Easy Vector. Colonies with inserts were screened by hybridization with the respective ^{32}P-labelled oligonucleotide (DeScenzo & Harrington 1994). Plasmid DNA isolation, sequencing of positive clones, primer design and labelling were as stated for anchor PCR. When necessary, the Universal Genomewalker kit was used to obtain further flanking sequences for primer design.

PCR amplifications of all microsatellite loci were performed using a 96-well thermal cycler (PTC-100, MJ Research Inc., Watertown, Massachusetts). Cycling conditions

Table 2 Microsatellite alleles, based on approximate band sizes as determined by GeneScan analysis, found in isolates of *Ceratocystis fimbriata* from sweet potato, sycamore and cacao. Number of isolates tested is shown in parentheses

Locus	Sweet potato isolates (15)	Sycamore isolates (67)	Ecuadorian–type cacao isolates (10)	Costa Rican–type cacao isolates (69)
CfAAG8	176, 188	176	173	188
CfAAG9	400	409	415, 418	409
CfCAA9	206	270, 276, 294, 312, 368, 386, 400, 410	157, 245, 254, 284	184, 247, 263, 277, 280, 286, 292
CfCAA10	136	127	133	130, 133
CfCAA15	323	288, 317	344	323, 326, 338
CfCAA38	151	134, 157	157	227, 246, 271, 273
CfCAA80	305	268, 291, 294, 297, 300	302	288, 308, 317
CfCAT1	257	260	257	260, 271, 274
CfCAT3K	323	323, 326, 329	326	329, 332
CfCAT9X	282	284/287*	279/281*	276/284, 276/287*
CfCAT1200	377	391, 394, 409	385, 388	377
CfCAG5	319	328	328	340
CfCAG15	176	317, 333, 337, 340, 343, 346, 353, 356, 359,365, 399	263, 287, 308	272, 275, 278
CfCAG900	196	196	193	196
CfGACA60	190	190	190	190, 196
CfGACA650	232	235, 256, 259, 276, 285, 290, 295, 300, 328	211	211, 220, 236

*The CfCAT9X primers amplify two products for most isolates, and unique combinations of the two product sizes are scored as a single allele.

were an initial denaturing step of 95 °C for 95 s, followed by 35 cycles of 94 °C for 30 s, 58 °C for 1 m, and 72 °C for 30 s, with a final extension at 72 °C for 30 m. Each reaction (20 μL) contained 2 μL of 10X reaction buffer (500 mM KCl, 1% Triton X-100, 100 mM Tris-HCL, pH 9.0), 200 μM of each dNTP, 4 mM $MgCl_2$, 5.0 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Promega Inc., Madison, WI), and 10–50 ng of RNase treated template. Before electrophoresis, the HEX, TET and FAM labelled products (0.5 μL each) from three separate reactions were combined and added to a master mix comprised of 2.4 μL formamide, 0.5 μL blue dextran, and 0.6 μL GENESCAN-500 [TAMRA] size standard (PE Biosystems, Foster City, CA). A final volume of 1.3 μL was electrophoresed on a 4.5% acrylamide gel on an Applied Biosystems (ABI) Prism 377 DNA sequencer. Project and sample files were generated and allele sizes determined using ABI GENESCAN software version 3.1 and GENOTYPER software version 2.5.

Sixteen polymorphic microsatellite loci were identified (Table 1) when tested against isolates representing four host-specialized lineages within the Latin American clade of *C. fimbriata* (Baker *et al*. 2003). Number of alleles observed per locus ranged from two to 20 (Table 2). In general, more alleles were found with microsatellite loci identified by the Edwards technique than by the anchor PCR technique. The low level of polymorphism found in the sweet potato lineage is probably due to the clonal spread of the pathogen on sweet potato storage roots from a single, unidentified population in Latin America (Baker *et al*. 2003). Most of the polymorphism within the sycamore and the cacao lineages was found among isolates from the southeastern USA and northwestern South America, respectively, the purported origins of these lineages.

The microsatellite markers developed here will be useful for studies of population structure and species limits within *C. fimbriata*. Their variability will also make them invaluable for tracking introduced strains.

Acknowledgements

This research was supported by the National Science Foundation through grant DEB-0128104. We thank Wei Chen and Nicole Wedemeyer for their technical assistance.

References

Baker CJ, Harrington TC, Krauss U, Alfenas AC (2003) Genetic variability and host specialization in the Latin American clade of *Ceratocystis fimbriata*. *Phytopathology*, **93**, 1274–1284.

Barnes I, Gaur A, Burgess T, Roux J, Wingfield B, Wingfield M (2001) Microsatellite markers reflect intra-specific relationships

between isolates of the vascular wilt pathogen *Ceratocystis fimbriata*. *Molecular Plant Pathology*, **2**, 319–325.

CAB International (2001) *Ceratocystis fimbriata* [original text prepared by CJ Baker and TC Harrington]. In: *Crop Protection Compendium*. CAB International, Wallingford, UK.

DeScenzo RA, Harrington TC (1994) Use of $(CAT)_5$ as a DNA fingerprinting probe for fungi. *Phytopathology*, **84**, 534–540.

Edwards KJ, Barker JHA, Daley A, Jones C, Karp A (1996) Microsatellite libraries enriched for several microsatellite sequences in plants. *Biotechniques*, **20**, 758.

Fisher PJ, Gardner RC, Richardson TE (1996) Single locus microsatellites isolated using 5′-anchored PCR. *Nucleic Acids Research*, **24**, 4369–4371.

Santini A, Capretti P (2000) Analysis of the Italian population of *Ceratocystis fimbriata* f.sp *platani* using RAPD and minisatellite markers. *Plant Pathology*, **49**, 461–467.

Toth G, Gaspari Z, Jurka J (2000) Microsatellites in different eukaryotic genomes: Survey and analysis. *Genome Research*, **10**, 967–981.

Wostemeyer J, Kreibich A (2002) Repetitive DNA elements in fungi (Mycota): impact on genomic architecture and evolution. *Current Genetics*, **41**, 189–198.